

24 June 1982

Making a strategy for Europe

The European Commission's heart may be in the right place in the support of science and technology, but it must learn patience. Europe will be there a century from now.

The European Community is going through a strange and unrecognized metamorphosis. President Ronald Reagan has come and gone, leaving behind him a trail of disappointment. What worries Europeans most is not that he was less than well-informed about their affairs but that so little happened or got done. The Falklands war has been settled, after a fashion, reminding Europeans that in the last resort blood, which means financial common interest, is thicker than water. However reluctantly, the members of the Community found that they had no choice but to go along with the unexpected British assertion of national purpose (and Irish diffidence on this point so to speak is the exception that proves the rule). Meanwhile, the whole of Europe is perplexed that there should be little it can do about the mounting crisis in the Middle East. There is almost nothing that can be done to avert the slow erosion of Chancellor Helmut Schmidt's government in Bonn. This and the concatenation of dismal happenings has, however, had a paradoxical effect — Europeans seem to have been convinced that they are in the same boat, and that they had better make the best of it. The constructive performance of the government of France at the Versailles summit, however eccentric, has been heartening for everybody on the east of the Atlantic. This, strange as it may seem, is the spirit in which the meeting of the council of science ministers of the Community this week should be judged.

The objective of the meeting is to make a further step towards a science policy for the European Community. It tends to be forgotten that the Community has had a science policy since before its own creation — a policy called Euratom. It is, however, well within living memory that whenever the Community has since tried to hammer out some concerted plan of action on its support of science and technology, the result has been conspicuously inconspicuous. Plans for coordinated programmes of research and development in computers, or telecommunications, have regularly foundered or have been still-born. The explanation is familiar but unremembered — national governments will not commit national funds to a common programme of research and development if they fear that their gain will be proportionately less than their financial contribution. To ensure that such an outcome will not arise, they insist that whatever cooperative programmes there may be should be tightly controlled by representatives of the Community's member states, which is a recipe for making sure that the programmes will be second-rate. Will this week's meeting have a better chance of succeeding?

This meeting (see page 619) is at least well prepared. The council of ministers of science has been deluged with paper. There is also a hefty background document to direct attention to the directions in which, on a kind of statistical-historical view of events, the wind is blowing (see *Nature* 17 June, p.528). The Commission's new proposals show that it has learned something from the

disappointments of the past quarter of a century; the intention now is that there should be a political decision about the fields in which Community funds might be spent in the interests of the Community as a whole, a kind of political assessment of more detailed plans for spending money in the selected areas and then, finally, the making of research grants by specialist committees. The procedure proposed has the obvious advantage of putting a distance between the politicians who provide the funds and those who ultimately decide how they should be spent. The distance, however, is unlikely to be great. The committees that spend the money will be creatures of the member governments, and their members not so independent that they will be able to snap their fingers at those who nominate them. The best to hope for is that the regime now proposed will repeat past mistakes less glaringly.

Before it is too late, there is another model that European governments should consider — the agreement signed last week between the principal research councils of the United Kingdom and the Netherlands (see page 616). Even this marriage of convenience has arisen more or less by accident, partly because the British and the Dutch share common scientific interests and frustrations and partly because they get on well with each other in a wider context. Moreover, the deal now struck by the research councils falls far short of being a full marriage — there is no suggestion that British funds should for the time being be used to support research carried out in Dutch laboratories by nationals of the Netherlands. Yet the benefits of even this arrangement are classically simple. In at least some respects, the community of ideas will be enlarged.

The lesson for the European Community in this tale is that it takes time — and decades, not years — to bring about a tangible sense of collaboration between naturally competitive creative people, and that the collaboration works well only when the people who decide what should be done are the people who will then do the work. The model is persuasive, but by no stretch of the imagination quick enough for the European Commission's purpose. How, the Commission will be asking, can the "technology gap" then be bridged?

In reality, there must be some middle road. For political reasons, the Commission seeks to invest in research, necessarily applied research, that will both strengthen the sense of community and yield some practical benefit in the short run. Notoriously, what it wants can be accomplished only in the sphere in which commercial companies are strong and even dominant. It would be better advised to back away from the goal of immediate benefit, seeking instead to improve the cohesion of the research community. The imaginative use of Community funds for helping people from member states to move more freely within the Community would be a start, especially if the scope of such a scheme could be enlarged beyond the fields now cultivated by the European academies and by the European Science Foundation. Modest grant-making through independent bodies of trustee-like dignitaries would help still further if the grants found their way to those who in the judgement of their peers deserved them. Such painstaking work cannot, of course, be courted on to close the technology gap by some arbitrarily chosen target date, but no deliberate policy will do that (although luck might). But if Europe is here to stay, as the events of the past few weeks appear to attest, it will still be here a century or so from now.

US contributions

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Antarctic trouble ahead?

The welcome end of the Falklands war may yet herald trouble for the Antarctic Treaty.

The shooting may have stopped in the Falkland Islands but the state of war continues. Britain says that the exclusion zone in which Argentine ships will be regarded as hostile will remain in place around the Falklands until it is assured that hostilities are at an end. But the government of Argentina, in the throes of its post-war reconstruction, cannot yet make up its mind what should be done. Whatever government emerges in Buenos Aires, however, the Argentine claim of sovereignty over the Falklands is unlikely simply to be abandoned. The flat contradiction of that cry by the British government's insistence that the Falklands are a British possession — reinforced by the removal of an Argentine party from Thule in the South Sandwich Islands at the weekend — is a depressing omen for the future. The best chance that civility can return to Anglo-Argentine relations is that the issue of sovereignty should be put on ice, but the diplomats will be hard pressed to devise an agreement to that effect which each government can stomach. The prospect is that the negotiations that eventually take place will be as protracted and frustrating as those which preceded the war — and will be embittered because of it. The hope must be that this souring of relations will not spill over into the delicate negotiations now taking place under the umbrella of the Antarctic Treaty.

Britain and Argentina are key players in the circle of 14 nations that govern the region south of 60 degrees south latitude through the treaty, which came into effect in 1961. In the past few years, this group, building on a long history of mutual cooperation in the Antarctic, has begun a chain of decisions about how to dispose of the Antarctic region's food and mineral resources. Two critical meetings on these topics have taken place this month and it is a measure of the importance all parties attach to the subject that the meetings went forward despite the Falklands war.

The earlier meeting, at Hobart in Tasmania, was the first of the new commission established under the 1980 convention for the conservation of Antarctic marine living resources. Britain's scientific contribution, particularly that of Richard M. Laws, director of the British Antarctic Survey, has been important in establishing the concept that there is a serious risk that Antarctic krill — the region's principal food resource — could be overfished. Indeed, British research has shown that overfishing of some species of Antarctic fish has already taken place. Overfishing of krill (probably by the Soviet Union and Japan, which took perhaps as much as 500,000 tonnes last season) could imperil the higher life forms of the Antarctic that depend on krill as their main food source. Most endangered would be Antarctic whale stocks, already depleted by whaling earlier in the century. Formally, the commission could crack down on countries accused of overfishing, but the meeting in Hobart was also intended to establish a new scientific committee. This group is crucial to the success of the new convention, for it is the technical link that will determine when krill are being overfished. Since British research has contributed so fully to the understanding of Antarctic marine resources, it had been expected that Laws would become its first chairman. The fact that his election has now been vetoed is a clear sign of fall-out from the Falklands, and a warning that the amity of the treaty is in danger.

The second meeting, in Wellington, New Zealand, has been planned to make a start on the thorny question of the ultimate disposition of Antarctic minerals. How can exploitation be made compatible with the original treaty? The continent has huge continental shelves (the Weddell Sea floor is the size of Venezuela) with thick sedimentary deposits that may contain hydrocarbon deposits for example. One snag is that nobody agrees which country owns which part of Antarctica. Indeed, Britain, Argentina and Chile have overlapping and conflicting claims to the part south of South America, including the Weddell Sea. Whether it will be possible to allocate rights to mineral resources before the ownership of the land has been settled is problematical,

but there is no shortage of technical questions to be hammered out in advance — the technical steps that must be taken to avoid pollution of this unique environment for example.

The most obvious danger is that with the parties to the Falklands dispute also prominent signatories of the treaty, mischief-making will break out. Of claimants on the Antarctic, Argentina is among the most clamant, and has even sought to reinforce its claims by sending pregnant women to Antarctica so that their babies can be born Antarctic Argentines. Hitherto, Britain has been by comparison a modest claimant, saying that its formal claims in Antarctica are intended more as declarations of trusteeship than as old-fashioned territorial claims. It is to be hoped that the experience of the past three months will not have given the British too keen an appreciation of the pleasures of possession. Either way, since all decisions within the Antarctic Treaty must be taken unanimously, it will be easy for Argentina, Britain or some third party effectively to veto an unwelcome proposal by saying that the question cannot be settled until ownership has been resolved. By the convention signed in 1980, it has been agreed that something should be done to exploit marine resources sensibly, but nothing has been decided about minerals.

The meeting in Wellington has been made necessary by the need to forestall unwelcome activities in the Antarctic. The most obvious danger is that a failure to agree a set of rules will let in a host of cowboys. The best but also the most likely outcome is that there should be a moratorium on mineral exploitation until procedures have been worked out for telling how the profits (if any) should be distributed. But it should be fresh in the minds of member states that a very similar question occupied the conference on the Law of the Sea for the best part of a decade. It is not too soon to start on this negotiation.

Ships will go down

The Falklands conflict seems to have heartened the British naval lobby. It should not.

After its successful reoccupation of the Falkland Islands, the British government and especially the defence department finds itself engaged in a war that could politically or financially more damaging — a war with the most loyal of its supporters in the past three months, the members of the Navy lobby. Like the Falklands war, the war about naval policy has been rumbling away for months and even years, on one reckoning since in the 1960s the British government gave up the pretence of keeping a strong strategic force east of Suez and stopped building large aircraft carriers soon afterwards. More recently, however, the present government has had to forgo the services of one Navy minister (Mr Michael Steed) who resigned because he thought the British Navy was being starved of funds (Trident nuclear submarines not counting) while Mr John Nott, the present defence minister, appears to have succeeded Mr Francis Pym (by accident, now Foreign Secretary) because he showed more willingness to tell the Navy that they cannot have all the equipment they would like. Inevitably, in the weeks following a successful naval engagement, the case for building more ships is being loudly put.

It should be resisted and Mr Nott, to his credit, appears to be prepared to do just that. For the lessons of the Falklands war (which touch other modern governments than the British) run both ways. The task force to the Falklands could not have been mounted without ships, and large numbers of them, a large proportion of them fighting ships. That is one strike for the Navy lobby. But the recent conflict also showed that ships are vulnerable — part of the reason why successive British governments, whatever their first inclinations, have been cool about naval vessels as effective contributors to defence. (That they appear to burn so easily and well is nevertheless surprising.) Moreover, surface ships are likely for a long time to be more easily detected as floating metal objects on the surface of a foreign element, water, than they will be able to defend themselves with counter-measures. For the Navy lobby, that objection will not easily be turned.

US looks to biological weapons

Military takes new interest in DNA devices

Washington

New evidence suggests that the US Army is planning a substantial expansion in its biological warfare research programme, and may be particularly interested in the potential role of recombinant DNA in the development of biological weapons.

Since signing the 1972 Biological Weapons Convention, the US government has maintained that all research on biological warfare is unclassified and strictly defensive. This research is carried out openly at the US Army Medical Research Institute for Infectious Diseases (USAMRIID) at Fort Detrick, Maryland.

The Office of Management and Budget (OMB) has confirmed, however, that the Army has included in its overall budget for medical research a sum for defensive biological weapons systems which is classified and which is totally separate from the published budget.

The budget office apparently became concerned that the amount the Army requested for this classified work was out of proportion to the Army's stated aims in this area. One biologist contacted by OMB in the course of its review of the Army request says that it involved "hundreds of millions of dollars". USAMRIID's budget is approximately \$17 million.

Reports of the Army's interest in recombinant DNA appear to have originated from a request sent by the Army to the National Academy of Sciences several months ago. The academy has confirmed that the Army was sounding out its willingness to carry out studies on chemical and biological warfare that would involve classified materials. The academy's Assembly of Life Sciences decided not to participate in either classified work or any work involving biological warfare, but said it would consider doing unclassified studies on chemical weapons.

According to Professor Matthew Meselson of Harvard University, a long-time critic of the government's chemical and biological weapons policies, the topics the Army wanted the academy to study included the possible offensive uses of recombinant DNA technology in biological warfare, ostensibly for the purpose of better understanding how to defend against them.

The Army has since submitted a proposal to the academy for an unclassified study on the detection of and protection against mycotoxins, which the life sciences assembly apparently considers to be

chemical agents, despite their biological origin.

The public affairs officer for the Army's medical institute, Norm Covert, said he was not aware of contacts with the academy nor of any US Army research on biological weapons apart from that conducted at Fort Detrick. The institute's programme involves only the development of medical knowledge about biological warfare agents, including detection, treatment and prevention. The only project that uses recombinant DNA is an effort to develop an anthrax vaccine by cloning in *Escherichia coli* the determinant of the protective antigen for anthrax.

Two years ago the Army advertised in *Nature* for proposals to clone the gene for human acetylcholinesterase. At about the same time, the Army received permission from the National Institutes of Health Recombinant DNA Advisory Committee to clone the determinant of a mild exotoxin from *Pseudomonas*.

Concern over what the Army may be planning has prompted two researchers to propose an amendment to the recombinant DNA guidelines that would specifically forbid "the construction of biological

weapons by molecular cloning" (see *Nature* 17 June, p.527). It will be taken up at the next meeting of the Recombinant DNA Advisory Committee on 28 June.

In a statement from the Arms Control and Disarmament Agency which was cleared by the Department of Defense, the government told the committee that it has no objections to the amendment, but believes it to be unnecessary. The statement said that the Army's research programme "does not and will not involve research to create and screen 'new' organisms as potential biological warfare agents. Our research is, and will continue to be, limited to developing protective measures to recognized infectious agents which pose a biological warfare hazard." The statement also stresses that developing weapons for deterrence is not considered to qualify as one of the "prophylactic, protective or other peaceful purposes" for which research is allowed under the treaty. An official of the arms control agency said there was no evidence that the military was interested in going beyond defence research, and that the only classified material was information related to US vulnerability to biological attack.

Many of the same points were made in a

Changes for German cancer research

Heidelberg

The crucial meeting of the governing body (*Kuratorium*) of the German Cancer Research Centre seems to have passed off successfully on Monday (21 June). The centre will continue much on its present scale, but there will be substantial changes in the administration, thus vindicating the ambitions of Professor Hans Neurath, the late-director of the laboratory whose resignation last year precipitated the present crisis.

This week's meeting of the Kuratorium was called to consider the critical report of the independent commission of inquiry under Sir Michael Stoker that was published earlier this year and the response of the present staff, the new director and of the two governments (in Bonn and Stuttgart) that are involved.

The laboratory, strictly the *Deutsches Krebsforschungszentrum* (DKFZ), is West Germany's largest cancer research institute and has a staff of more than 1,000 deployed in 39 departments and a budget of nearly DM90 million (more than £20 million) a year.

The director, Professor Otto Westphal, and ministerial director Dr Fritz-Rudolf Güntsch presented the official response to the criticisms of the Stoker commission after the Kuratorium meeting. They agreed with the report's main contention, but said the commission had failed to appreciate the

broad aims of the institute and also the administrative and legal constraints of West German institutions.

Within three months, Otto Westphal has succeeded in obtaining the cooperation of the Bonn ministry, the *Land* and the members of the institute in reaching a consensus on new measures. First, the role of scientists in the running of DKFZ is to be strengthened. A new scientific committee manned from outside will advise the Kuratorium on scientific projects, staffing, space problems and personnel. The Kuratorium will have eight scientific members out of 14 and, it is hoped, will be less concerned with administration and more with science. The executive committee of DKFZ must now either implement or rediscuss all decisions of the Kuratorium and not, as in the past, leave them in abeyance.

Second, deficiencies in reviewing procedures will be remedied, in particular by *ad hoc* commissions appointed by the Kuratorium and under the chairmanship of a Kuratorium member.

DKFZ is one of the few big cancer institutes without its own clinic. Westphal admitted the Cinderella role of clinical research in West Germany, which he put down to the structure of the medical institutions. He is optimistic about opportunities for unconventional extra-institutional collaboration. **Sarah Tooze**

separate statement filed with the committee by Dr William Beisel, deputy for science at USAMRIID and the Defense Department's representative on the committee. An open question, however, is whether USAMRIID and the arms control agency are even aware of the Army's classified programme on defensive biological weapons. **Stephen Budiansky**

UK-Dutch agreement

Seeing stars

A far-reaching agreement for collaboration on major research projects was signed last week between the British Science and Engineering Research Council and its opposite number in the Netherlands, Nederlandse Organisatie voor Zuiver-Wetenschappelijk Onderzoek (ZWO). The immediate objective is to specify the rules under which the two research councils will collaborate on projects which involve expensive capital equipment.

A spokesman of the British council said last week that this development is a mark of the "steady convergence" of the policies of the two councils. Exactly a year ago, they signed an agreement on collaboration in astronomy under the terms of which ZWO will pay a fifth of the cost of the Las Palmas Observatory on Tenerife, receiving a fifth of the observing time in return.

Those administering the agreement say that the constructive benefits of the agreement are already apparent. Technical arguments by Dutch astronomers, for example, have led to the decision that the planned sub-millimetre telescope planned as part of the Las Palmas Observatory should be sited instead in Hawaii.

The intention now is that similar arrangements should be extended to other expensive projects, including the synchrotron radiation source at Daresbury, the British Starlink system for the common processing of astronomical data and possibly even the common use of major computer facilities. There is a possibility that the two councils may mount a joint project to build an improved neutron source for diffraction and other studies.

One administrative convenience in the new agreement is that it will not always be necessary for the costs of projects to be shared out one by one. Rather, when it suits the two councils, barter may replace the exchange of money.

The love affair between the two research councils has already led to the setting up of committees of which British and Dutch nationals are members. This has not yet, however, led to cross-membership of the principal policy-making committees, nor is there an immediate prospect of common grant-making procedures except where these are ancillary to some major project.

The Science and Engineering Research Council is the largest of the five in the United Kingdom, and exists to support scientific research at British universities

Cetus goes begging

Standard Oil Co. of California, an investor in Cetus Corporation, a leading California biotechnology firm, has elected not to fund the firm's plan to produce fructose commercially. Cetus hopes to find some other sponsor for the work, perhaps a sweetener manufacturer, instead.

The process would attempt to produce pure fructose at the same price as, or more cheaply than the main competitors — high fructose corn syrup (used in soft drinks) and sucrose. Although Cetus carries out research involving recombinant DNA techniques, the enzyme at the basis of the process was found using standard microbiological techniques.

Standard Oil's decision may represent a retrenchment by major oil companies in the biotechnology field generally. Investors in such firms are said to be more cautious now than they were one or two years ago, the budding recombinant DNA industry benefited from the enthusiasm — and dollars — of major firms. Standard Oil of California (Socal) owns 17 per cent of Cetus Stock.

Deborah Shapley

and polytechnics. The terms of reference of ZWO are wider, extending to the humanities and social sciences. On the other hand, ZWO is not responsible for Dutch contributions to international projects (such as CERN), while the proposed Anglo-Dutch collaboration in the infrared satellite is similarly the direct responsibility of the Dutch government.

As a rule of thumb, the budget of ZWO in Dutch guilders is roughly equal to the budget of the Science and Engineering Research Council in pounds sterling (£1 = 4.7 guilders).

Global systems analysis

Insult or injury?

The International Institute for Applied Systems Analysis (IIASA) in Vienna is going hawking for money. Abandoned recently by the US National Academy of Sciences, IIASA has suffered another blow. Its British member, the Royal Society, quit last week. Hopes for survival now centre on the institute's friends in the United States, who are trying to raise private money. Next month the director, Canadian Professor C. S. Holling, will visit Britain to attempt to rekindle interest.

For just over a decade, the institute's chief claim on public attention has been its unique status as the paradigm of the truly East-West research centre. Its collapse, now possible, could spell the end of an era or simply mean that the project was misconceived.

IIASA is being attacked on three fronts: political, financial and academic.

Politically, the institute is a creature of detente, planned as a forum where East and West could tackle problems of global importance; but detente is dead, as is the Reagan government's interest in IIASA. And the £5 million budget (1982) is seen as an unnecessary luxury in hard times.

Academic criticism such as that which seems to have moulded the British decision has been more of a surprise. Sir Hermann Bondi, British member of the IIASA Council and also chairman of the UK National Committee for IIASA under the Royal Society, professes himself "horried" at how few friends IIASA has on the committee. But Sir Hermann is by background a theoretical physicist, not a systems analyst, and must demur to professional opinion.

The UK committee seems to have been dismayed by the draft research plan for 1983, the first produced by Professor Holling. The committee had expected to see drastic pruning of the 24 projects current in 1981 under Soviet leadership of IIASA. Holling had reduced the number to nine, but these included the biggest of last year's projects, including for example the analysis of energy policies, the impact of industrial change, environmental regulation and institutions and regional and urban development. The committee decided that resources at IIASA were still being spread too thinly.

This opinion thus left the Royal Society unable to press the British government to continue membership. The Department of the Environment, the formal channel for the annual subscription, had already decided for internal reasons that it need no longer support IIASA. The Royal Society, Britain's formal member, without any obvious means of paying the subscription, resigned.

IIASA complains that the British committee does not understand the institute's objectives, which — as Holling has put it — are to provide policy-makers with "creative options". The British attitude is more pragmatic, IIASA staff members say, while there is very little overlap between the research interests of UK National Committee members and those of IIASA.

The institute is also offended that the British decision was taken when only the draft plan for 1983 was available. With the plan approved by the IIASA Council, the proposals can now be "fleshed out". Bondi has invited Holling to present his more detailed case personally to the UK National Committee next month. Holling will travel with Professor K. S. Parik of India and A. Wierzbicki of Poland to emphasize the international interest in IIASA work. It is too late for the Royal Society to reverse its decision, but Bondi hopes it is not too late to find another sponsor, however slim the chances.

In the past, IIASA has been represented by member institutions from 17 countries including the Soviet Union and the United

States which had "Class A" membership and each paid 36 million Austrian schillings (£1.2 million). Lesser Class B members paid 5.4 million schillings. IASAs's nine projects for 1983 will now have to be found within a budget of 115 million schillings. IASAs's friends in the United States are looking for \$1–1.5 million a year from 1984.

Robert Walgate

Commercialization of research

So far. . .

Boston

Biological research progresses much more slowly than Wall Street transactions, Dr Walter Gilbert, professor of molecular biology at Harvard University reminded an audience of senior businessmen and brokers worth many millions in venture capital last week in the last lecture of his illustrious academic career. Gilbert, who in two weeks will take up full-time duties at the biotechnology firm Biogen, joined other eminent scientists in giving the executives a day-long, \$300 per head tutorial on the rudiments of biotechnology. Several speakers addressed the question of how involved a university can get in the big business of biotechnology and still retain its integrity and autonomy.

Gilbert described the 30 years of basic research that prepared the ground for the recombinant DNA boom. Only in 1978, after three decades of work, could scientists attack the problem of making particular genetically engineered commercial products; and even the applied problems are proving very slow to solve. After the meeting, Gilbert reflected at length on the issues of the conference.

In the first place, Gilbert stated that aggressive university patent and "technology transfer" programmes are in fact superfluous. In the United States there exists no real technology transfer gap. "This is evident in the plethora of small biotechnology companies, and is due wholly to the effervescence of America's venture capitalists", he said, "who are the missing link in England."

Gilbert also took issue with the public perception that applied research is the intellectually weaker sister of basic research. And in fact, Gilbert continued, although everyone sees basic research as the wellspring of new scientific ideas, this is not exclusively true. "One reason universities encourage their faculty to consult for industry is that they realize that this is an important way for teachers to learn about the problems in the world."

With a foot in both industry and academia for five or more years now, Gilbert is in a unique position to comment on the threat to a laboratory of such cases as his own. Because "the student has absolutely no notion of why a professor puts him on a scientific problem", the threat of student exploitation is real. But Gilbert thinks that any commercial exploi-

tation of a student will be exposed. In any case, the transgressor will be doomed, since commercially motivated work is often not academically meritorious and so he will not attract top-flight graduate students and government grants.

Dr Derek Bok, president of Harvard, gave the conference the clearest statement so far of the line universities will be taking on relations with biotechnology companies. Bok's "cleaner than thou line", as Gilbert later called it, was drawn up at conclaves of university presidents such as that last March in Pajaro Dunes (*Nature* 1 April, p.381). Universities are generally eager to aid in closing the putative "technology transfer gap", Bok said. He encouraged so-called "bilateral research agreements", such as the \$50 million agreement between Hoechst and Massachusetts General Hospital. He underlined as justification for his position the current shortage of public research funding, the liberty inherent in a multiplicity of research sponsors and the fact that private support demands of the investigator little of the red tape that a federal grant does. Bok stated four provisos for such agreements: (1) that the sponsor cannot stipulate what the scientist studies; (2) that bilateral agreements must be published; (3) that a firm must guarantee a discoverer's right to publish his findings; and (4) that a firm must get preferential patent rights only when it has clearly funded the work involved.

Gilbert later called the patent issue the messiest in all the debates. Patents on discoveries made by university workers should go to the discoverer himself, not to a private supporter and particularly not to the university. "The superficial rationale behind Harvard's current position of retaining patent rights on its employees' inventions is that this is in the public interest", Gilbert said, "but actually the university has only one motive: remuneration." In Gilbert's view, the university should renounce patent rights and any other procedures which would lead to its motives appearing suspect. The disinterestedness that would result is one of the university's strongest assets. In the long run, renouncing patents would actually make it richer, since over the centuries such a climate of disinterestedness is what has made universities worth endowing.

Bok overtly proscribed investment by the university in a firm in which one of its faculty members had a stake. He recounted the example of the firm in which Dr Mark Ptashne is involved, in which Harvard once considered a joint venture. Although it "takes an imaginative soul to dream up a gift a university will refuse", Bok mused, this was an offer which Harvard had to turn down to preserve good public relations, faculty morale and general academic values. Dr Ptashne later retorted that in fact Bok had got it wrong — that it was the university and not Ptashne's company that made the first overture.

Cases in which a faculty member has a stake of money or time in a biotechnology firm are the hardest to legislate. The concern of Harvard, Bok said, is with "what occupies a professor's mind when he wakes up in the morning". Since this information is inaccessible, there must be certain guidelines. For instance, at Harvard and at Massachusetts Institute of Technology no professor may be a corporate executive and retain his faculty post. Nor may he hold a "significant number of shares" in a job-related company. ("The main flaw in Bok's position," Gilbert later said, "is in his argument on shareholding. What constitutes a 'significant number of shares?' There is only one clean line and that is no shares at all.") Bok said that on the other hand the university permits its faculty members to spend up to 50 days per year away from their laboratories consulting with industry (at a going rate of \$200–\$2,000 per day). He warned that it is as important that a university should be seen to avoid the dangers in faculty or university relations with biotechnology firms as that it should actually avoid them.

James Aisenberg

British biotechnology

Imperial poised

Imperial College London has found an ingenious way of dragging itself by its bootstraps into the brave new world of biotechnology. A few weeks after the college arranged that its fermentation pilot plant should be transferred to a private company financed by TDC Developments Limited and called Imperial Biotechnology Limited, a venture capital firm, the college is in the market for three staff members — a professor (whose stipend will be provided by the Leverhulme Trust) and two lecturers, whose salaries will be met by the fees earned from contracts with outside bodies based on the use of the pilot plant.

The chairman of the Centre for Biotechnology that will result, Professor Brian Hartley, says that the development at Imperial College shows that universities can still embark on novel undertakings when general funds for universities are restricted. In these days, he says, a college that has three vacant posts on offer in such a field is in a unique position. He is undismayed that the local branch of the academics' union (the Association of University Teachers) has complained that new academic posts should not be created when the holders of other academic posts are being threatened with redundancy.

The Imperial College pilot plant, built somewhat before its time was ripe at the instance of the late Sir Ernst Chain and until quite recently considered something of a white elephant, and has turned out to be a marketable asset. Imperial College has, however, reserved 20 per cent of the time available at the pilot plant in lieu of shares that would otherwise have been available.

Exploiting genetics

Washington

Congress keeps muttering about legislation to regulate links between universities and industry on the exploitation of genetic engineering. Last week, Congressman Albert Gore's oversight committee held two days of hearings on the conflicts of interest that have arisen or may arise. The occasion gave university representatives an opportunity to parade local solutions they believe will prevent a recurrence of recent controversies.

In several well-publicized cases, university researchers pursued work in outside corporations that closely paralleled their academic research, and the financial arrangements between the parties, suggested conflicts of interest. (In the recent Calgene case, for example, a plant biologist at the University of California at Davis received a grant from Allied Chemical. The researcher was also vice-president of Calgene, a biotechnology firm in which Allied subsequently purchased a 20 per cent interest (see *Nature* 4 March, p.6).

Recent large grants by corporations to Massachusetts General Hospital and Washington University, St Louis, have heightened concern over possible conflicts with traditional freedom of academic inquiry.

Roderick Park, vice-chancellor of the University of California at Berkeley, testified — as did representatives of the University of Wisconsin, Stanford University and the University of California at Davis — that the universities are themselves concerned. Draft principles under consideration at Berkeley, for example, would prohibit

any research on campus "whose benefits to education and research are small"; would require sponsors of research to allow free publication of all results; and would "scrutinize", but not necessarily forbid any arrangement that involved sponsorship of campus research by a company in which the researcher held a financial interest.

At the hearings, critics of university-industry ties had a chance to point a finger at some of the more notorious conflict-of-interest cases and called for federal guidelines on financial disclosure. Albert Meyerhoff of the Natural Resources Defense Council (NRDC) testified that the universities have already proved incapable of confronting these issues themselves. The Pajaro Dunes conference failed to accomplish its purpose of drawing up ground rules for industry-sponsored research, he said. Meyerhoff also criticized the Stanford faculty for having rejected a proposal requiring disclosure of conflicts of interest.

NRDC called for federal legislation that would require universities receiving federal research funds to adopt financial disclosure rules for faculty. Researchers should disclose any financial interests they have in research sponsors and also any interests in companies that could benefit from their research, NRDC said.

No such legislation has been introduced. But Representative Albert Gore (Democrat, Tennessee), who conducted the hearings, has been increasingly concerned over the effects of new industry-university ties.

Stephen Budiansky

are also required "during (their) visit and afterwards . . . not to mention the Council's name in any public controversy". Mr Alderson says that the last condition is inspired by Section 2 of Britain's Official Secrets Act and contravenes the human rights guidelines of several international organizations.

The issue may be taken further. Not only does Mr Alderson plan to write to the national newspapers but Lord Avebury has written to Sir James Gowans, Secretary of MRC, drawing his attention to Form Y. Lord Avebury professed himself astonished that "any self-respecting scientist would give such an undertaking which on the face of it puts a gag on visiting scientists" and prevents them from ever commenting publicly on the policy of the research councils.

The research councils are clearly surprised and bewildered to find themselves at the centre of such a maelstrom. "Form Y? Notes for the guidance of visiting scientific workers? What is it? We've never heard of it." Form Y is in fact used only by MRC; visiting scientists are asked to sign it in exchange for access to MRC facilities.

An MRC spokesman said that Form Y had nothing to do with the Official Secrets Act and merely expressed work conventions understood by employees of any establishment. The phrase which requires that MRC should not be mentioned in any public controversy conforms with the guidance given to its own employees and was inserted to avoid embarrassment — when making a public statement the individual should make it clear that he is speaking on his own behalf and not stating council policy unless he has obtained official approval in advance. MRC employees, he claimed, are not prevented from expressing their opinions as private individuals.

Visiting scientists at ARC and the National Environment Research Council (NERC) are asked to sign a form covering standard conditions — patent rights, health regulations, publication conditions — but there is no clause referring to the councils in any public controversy. NERC employees and overseas visitors who come for two or three months do have to sign Section 2 of the 1911 Official Secrets Act and Section 1(2) of the 1920 Act. SERC has no official form at all, and the only restriction on visitors is that covering patent agreements.

What seems clear is that the obligation, explicit or understood, on an employee not to speak publicly on behalf of his employing body without official approval does not limit his right to speak out on any issue whatsoever as long as he makes it clear he is expressing a private opinion. What is not so clear, however, is whether an individual, employed by or visiting a research council, can publicly criticize the policy of the council without the fear of being disciplined.

Jane Wynn

Professor Hartley says that the new research and teaching centre will have two chief lines of enquiry — the engineering of microorganisms able to digest wood and wood-like natural materials (including sugar-cane and bagasse) into usable chemicals, and the development of enzyme electrodes or sensors by which means the activity of a biochemical enzyme can be coupled directly to a semiconductor device. "We may be able to make microprocessors that can smell", Professor Hartley said.

On his own position at the centre, Professor Hartley says that there is no conflict between his chairmanship of a new academic centre and his membership of the scientific advisory board of Biogen, the Swiss-based company. He explains that his contract with Biogen allows him to keep confidential his work within Imperial College and vice versa. He considers that the centre, now that it is a going concern, will be able to apply successfully for a subvention from the fund earmarked by the University Grants Committee for the support of biotechnology in British universities (see *Nature* 20 May, p.173).

Research council visitors

No-gag gag

Allegations that foreign scientists visiting British research council laboratories are subject to serious constraints on their freedom of expression seem to be a storm in a teacup. What is at issue is whether the terms on which visiting scientists agree to work in British laboratories muzzle public criticism of council policy.

Mr Stanley Alderson, who describes himself as a writer and human rights campaigner, claims that foreign scientists visiting the Agricultural Research Council (ARC), Medical Research Council (MRC), Science and Engineering Research Council (SERC) and Social Sciences Research Council (SSRC) are asked to sign an agreement, known as Form Y, accepting the conditions of work undertaken by British employees of the councils. As well as agreeing to observe safety arrangements, patent regulations and conditions governing publication of research work, visiting scientists

Plans for research

Europe awakes

Brussels

"It is of course the duty of the European Commission to be ambitious", commented one British official cynically on the European Commission's new plans to increase EEC science cooperation. Next week's council meeting of the research ministers (on 30 June) will be sitting in judgement on a strategy for the future of European science policy, the fruit of two years of conferences and discussions that followed the 30 May mandate of 1980, when Mrs Margaret Thatcher won Britain's refunds from EEC's budget and when the other nine member states agreed to strengthen and broaden EEC's activities in the industrial, social and scientific fields.

Step by step, the European Commission has been subtly building up momentum by calling unusually frequent research council meetings during the past nine months. Previously, research ministers met every two years but, pushed by the European Commissioner with the science portfolio, Vicomte Etienne Davignon, the EEC ministers met in November and March and will see each other next week and again in the autumn.

The momentum for change is being created not so much by decisions as by ministerial debates intended to set the mould for a recasting of the priorities and design of EEC research programmes. The meetings so far have established the important role of research and development in achieving industrial competitiveness with the Japanese and Americans in new technologies and the economic and social dangers of failing to do so.

Davignon wants a new research and development programme with five objectives: improving the competitiveness of agriculture and industry; the management of raw materials and energy resources; the quality of life in terms of health and safety and the environment; helping the less developed countries; and stimulated science and technology in the Community. EEC spending — 645 million European currency units (£1,150 million) in the 1983 budget — represents only 2.7 per cent of the budget as a whole. Sixty-nine per cent of the total budget goes on energy, 13.5 per cent on industrial competitiveness, 9 per cent on the quality of life, 2 per cent on agriculture and 1 per cent on raw materials. But research and development employs more staff (3,000) than any other directorate general in the Commission if the staffs of the Joint Research Centres are included.

The new strategy would be based on five massive long-term programmes. One of these, thermonuclear fusion, is already in operation. The others would tackle information technology, biotechnology, the stimulation of industrial and agricultural competitiveness and research intended to

aid developing countries.

The broad outlines of the information technology programme, dubbed "Esprit", have already been sketched out (see *Nature*, 3 June, p.352); work on the other programmes may begin after the forthcoming council meeting. The reason for this is significant. Instead of laboriously budgeted and detailed programmes being presented to infrequent ministerial meetings, the planning would in future work from the top downwards.

There would be a new body, the Committee for the European Development of Science and Technology (CODEST) bringing together twenty of the great and the good in the world of science, technology and industry to select research objectives. It would interpret a master plan agreed by ministers, who would specify in advance the resources available. A plan for encouraging the research judged necessary would then be devised by CODEST and the Commission, on which basis proposals from research teams capable of achieving the objectives in question would be chosen. But independent experts drawn largely from industry would actually run the programmes. The first steps towards a long-term programme would be pilot projects funded by EEC.

In this way, with CODEST operating as a think tank, the Commission as its administrative arm and the day to day running handled independently, the political rows that sully programmes such as the reactor safety project Super-Sara would be avoided. There would no longer be haggling and long delays over the budgets of programmes that had already been worked out in detail.

The realization of the Commission's plans depends on the momentum established by the forthcoming council meeting. It is suggested in Brussels that Davignon will continue merely to be humoured because in a time of budgetary restraint EEC research, whatever other purposes it may fulfill, is not on its past record the best value for money. Few deny that there is a drastic rethinking of the rationale behind European research and development.

Jasper Becker

Soviet mathematics

No more sets

The Soviet Union is to introduce a new mathematics syllabus in the senior classes of secondary schools. The objective according to Dr M.I. Kondakov, president of the Academy of Pedagogic Sciences, is to resolve the major debate which has been raging among Soviet educationalists since the introduction of the present syllabus into Soviet schools a few years ago.

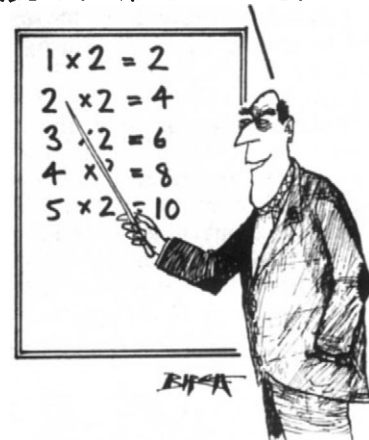
The new syllabus was part of a major overhaul of school education, begun in the mid-1960s. Special emphasis was placed on an adequate grasp of general principles and on pupils' ability to reason for themselves.

Learning by rote was to be replaced by an increased amount of independent work. In particular, mathematics was to be firmly based on set theory.

Initially, the new syllabus appeared to be a success. In the first three school years the proportion of poor achievers in mathematics dropped from 15 per cent in 1967 to 3 per cent in 1975. But in the middle and senior years, the situation was less happy. In 1977, when the first batch of students to have experienced seven years of the new syllabus took their school-leaving examinations, it was found that a majority had failed to grasp the new concepts.

One particular failing was a marked lack of spatial imagination, attributable either to the introduction at too early an age of a purely academic approach or else to the fact that, so far from working "independently", the pupils were issued with ready-drawn diagrams to save time in class. The

"NO MORE DECADENT
WESTERN MATHS FOR US!"



curriculum seems also to have nullified the avowed purpose of the reforms by using a different terminology from that used in science classes, so that pupils found themselves unable to solve quadratic or trigonometric equations arising in physics classes.

These examination results triggered a major debate in the Soviet educational press, with the president of the Academy of Sciences, Dr Anatolii D. Aleksandrov, and the prestigious mathematician L.I. Pontryagin criticizing the syllabus. Their equally prestigious colleagues S.L. Sobolev and L.V. Kantorovich, however, cautiously supported it.

The debate raged for two years (a detailed analysis of the arguments appears in the current issue of *Journal of Curriculum Studies*), and, perhaps significantly, teachers' complaints centred around difficulties in implementing the syllabus (lack of classroom aids and teachers' handbooks) rather than the actual content, although their main difficulties seem to have arisen from the highly theoretical orientation of the syllabus.

Parallel with this debate, the weekly *Literaturnaya Gazeta* launched its own

discussion, which ran for some months and in general proved to be critical of the current emphasis on mathematics as such. One correspondent depicted a future with a computer on every classroom desk and suggested that with the advent of the computer as "co-respondent", the long "marriage" of mathematics and science was ripe for divorce.

Professor M. Evgrafov, of the Mathematics Institute of the Academy of Sciences went further. "Not a single result" in pure mathematics, he claimed, had had any practical application for the past fifty years, and the current insistence on a mathematical training for scientists was "wanton", "rigid" and "useless". In the future, he said, problem-solving will be increasingly left to programmers whom he described as "persons of middle-level qualifications".

In 1980, Pontryagin shifted the whole debate from the educational and cultural journals to *Kommunist*, the leading Party theoretical monthly. His criticisms thus took on the force of tacit party policy, and have undoubtedly played a part in shaping the revised syllabus, which will give greater emphasis to practical applications and seems designed to train the "middle level" technicians of whom Evgrafov spoke.

The signs are that the new syllabus will downgrade set theory (except, optionally, in geometry), eliminate "difficult" terminology and symbolism and play down theoretical precision in favour of an "intuitive and descriptive" approach with an emphasis on new mathematical technology, including computers. **Vera Rich**

British Antarctic Survey

A good wind

On the principle that "it's an ill wind . . .", the British Antarctic Survey is hoping that the war over the Falkland Islands will bring back good times again. Meanwhile, the survey is congratulating itself that those of its scientists left stranded on South Georgia after the occupation by Argentine forces have made good use of their extra six weeks on the island.

The supply vessel that would normally have picked up the scientific parties from South Georgia in April, the RRS *Bransfield*, also seems to have done useful work. The ship was apparently in the Chilean port of Puerto Arenas at the occupation of the Falkland Islands, collecting spares for diesel engines and other gear. In the succeeding weeks, it was able to monitor radio transmissions between Port Stanley, on the Falkland Islands, and the mainland of Argentina.

During the ship's spell in Antarctic waters (below 60 degrees south), the ship served as a relay station between the British bases in Antarctica, and even kept in touch with those isolated on South Georgia by means of relayed signals from the base at Signey one the South Orkney Islands.

Falkland minerals

An agreement of sorts between Argentina and the United Kingdom on the title to submarine mineral rights off the Falkland Islands appears to have been reached on the eve of the occupation of the islands, and to have been embodied at the end of April in a resolution of the United Nations conference on the Law of the Sea. The resolution adopted by the conference (but with Argentina's dissent) says that where there are disputes about the sovereignty of territories (such as the Falkland Islands), mineral resources shall be exploited as if the wishes of the inhabitants were paramount.

This resolution appears to be a compromise reached after the rejection by Western powers, France and the United Kingdom in particular, of a draft article of the treaty that would have given each disputing party an effective veto on mineral exploitation, and which used the weaker form of words "for the benefit of the inhabitants".

The rejected article of the treaty, described as "anti-colonialist", would also have given the United Nations an important role to play in the regulation of disputed marine mineral rights. The resolution eventually adopted by the conference, which will not have the force of international law, plainly suits the British book much better.

Normally, communications with the British Antarctic bases are relayed to Cambridge, England, through the radio and telex station at Port Stanley, out of action since early April.

For most of the Antarctic programme, the interruption caused by the Falklands war should be small, as most of the bases were resupplied just before the occupation of the Falklands. On South Georgia, however, where there has been a weather station in continuous operation since 1944 and more recently a summer biological station on Bird Island nearby, a season's work is likely to be lost.

Apart from the difficulties of operating its two ancient ships in hostile waters, most of the British Antarctic Survey's problems are monetary. The numbers of people on its books have been shrinking for the past decade, and now amount to about 300, of whom 120 are scientists. The budget for the current year is about £6 million, a modest decline compared with the previous year without allowing for inflation. Even so, it has been possible to rebuild two of the three bases on the Antarctic Peninsula, while there is an ambitious plan to rebuild the base at Halley, on the Weddell Sea.

What the survey now hopes is that the British government's renewed interest in the South Atlantic, and the practical need to find peaceful activities that will occupy British nationals there, will prompt more generous budgets.

Chinese census

First head-count begins next week

Experts applaud revised plan

Washington

The only modern census of China's estimated 1,000 million people, who make up one-quarter to one-fifth of the world's population, will take place on 1 July. China's last census was in 1964, but Western demographers consider its results suspect. The earlier count in 1953 is similarly in doubt. So the July count is a demographic event of major historical proportions.

At the 1953 census, Mao Tse-Tung officially governed 583,603,417 people. The number was growing rapidly in accordance with Chinese family customs and Mao's dictum of 1949 that "revolution plus production can solve the problem of feeding the population . . . Of all things in the world, people are the most precious . . .". But by 1957, the net increase was 20 people per thousand per year and the official line changed. Mao said the population should be stabilized, perhaps at 600 million people.

That figure had probably already been exceeded and subsequent famines, political upheavals and other changing conditions have increased uncertainty as to how many people China has. Age, sex and regional breakdowns are critical for its centralized state planning. But an accurate count, besides helping the government, will among other things show whether the government's past claims for success in population control are true.

But the scale of the enterprise is staggering. How do you count 1,000 million people? Previous censuses in China were carried out literally with hand tabulation and abacuses. For the 1982 census, the government has acquired 20 IBM 4331 E Series computers and one IBM 4341 computer with funds from the United Nations Fund for Population Activities (UNPA), and has bought eight Wang computers on its own.

The five million or so enumerators will be office workers, staff of local institutions, rural accountants and record keepers. In a test count in the county of Wuxi, enumerators were required to have at least a junior middle school education. The census forms will have 19 categories to fill in, some by code, some by ordinary writing. (The 1964 census form had eight categories; the 1953 census had five). Representatives of each household will be summoned to registration points to tell the enumerators how many people were in the household at midnight, 1 July. Or they will be reached by mail, or visited on their

boats. Post-enumeration checks will be made, followed by hand tabulations, after which the information will be sent into the computer for the region. Eventually the information will be centralized in Beijing. The schedule requires enumeration in early July and a provisional count by October 1982 of total population, numbers of males and females, population by province and other features. A machine tabulation of 10 per cent of the national sample will be ready in the spring of 1983, the government says, while the remaining tabulation could be completed and released in 1984. The Chinese are taking a pragmatic, self-critical approach to the census. This reflects not only the temper of the current regime but the tastes of the census's chief architect, Li Chengrui, head of the State Statistical Bureau (SSB). He and his colleagues have been collecting advice and assistance from outside China for some years now. They have changed their plans to provide the most modern count possible and say they will make the results fully available.

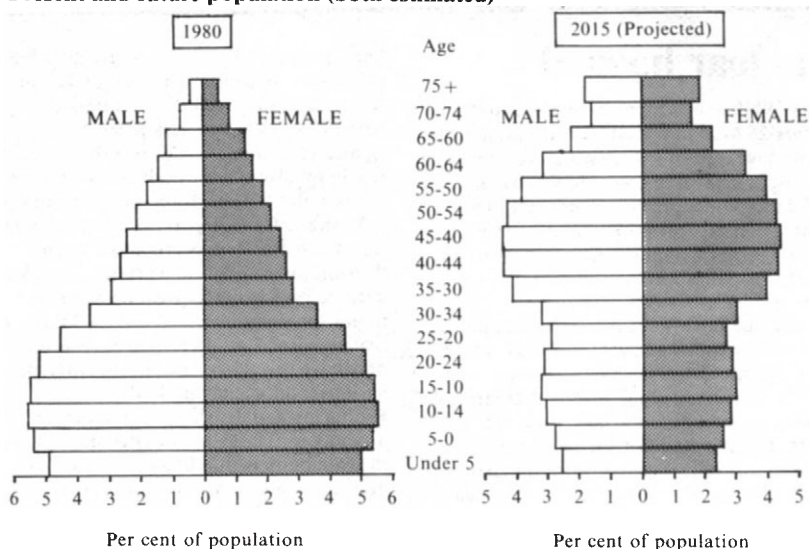
Credence

If the preparations for the count on 1 July are breathless, China was even less well prepared in 1979 for the census it then planned for 1980. Western demographers were surprised at the credence placed by the Chinese in their earlier counts, in 1953 and 1964, and critical of the plan that the same techniques should be used again. They were especially sceptical of the claimed error rate of 0.0014 per cent.

What seems to have been learned in the past few years of international consultation is that the Chinese government needs to know accurately how many people it has to govern, but that there are limits to the accuracy of any census. Their project may be helped by the character of Chinese society: Chinese neighbourhoods are close, while party officials know who lives in this or that house.

The cities will be the hardest problem. Many city dwellers have moved there illegally, and may remain hidden from the counters. There is also a risk that families that have broken family planning rules may

Present and future population (both estimated)



Source: *Intercom* Vol. 9, p.13 (Population Reference Bureau, August 1980).

conceal the fact, while local officials knowing that too many babies have been born in their districts may conspire to keep young children off the forms.

Even if such problems of bias can be overcome, the 1 July census will, for the time being, stand on its own. The earlier counts in 1953 and 1964 are known to have been inaccurate, and so inaccurate that they cannot be extrapolated forwards to 1982. Thus it will not be possible to assess the effectiveness of the family planning campaigns begun in 1956, especially because the Great Leap Forward and the succeeding Cultural Revolution made a hash of many state statistics. One difficulty during that period was the doctrine that statistics are "cold" and "too professional" and the accompanying injunction that statisticians should submit their work to the scrutiny of their superiors and should "be humble" in accepting corrections to it.

Part of the interest in the new census and in the age structure of the Chinese population it will provide is the light it may throw on events such as the widespread famine that in 1960 eliminated countless numbers of the population, and which may be apparent in the size of the age group that will be 22 this year. But the sheer size of the Chinese population will be the chief interest. Sample counts suggest that it is 1,000 million, but some US experts think that an underestimate by about 200 million.

Publicity

This year's census is being conducted very differently from that in 1964, whose existence leaked out only when a British cancer study cited figures from the previously unknown census. This time, the project is being undertaken in what amounts, for China, to a glare of publicity.

A major point of interest in the 1982 census is what it will tell about the success of recent government population control

measures. The crude birth rate stood at 37 births per thousand until Mao's first efforts at population control; it dropped to 20 per thousand in 1960, and rebounded up to 43.6 per thousand in 1963. The more serious controls of the 1970s have brought this rate down: in 1978 the Chinese claimed the crude birthrate was 18.34 per thousand. So, when the results are published in October, Western experts will study them closely to see how many people turn up in the 0 to 4 and 5 to 9 age group. The figure reflects the importance of population control measures to China. It represents how the Chinese population might change by the year 2015 if each woman in her life had an average of 1.5 babies. The comparison with China's traditional age profiles, on the left, show how important demographics can be for state political and economic planning.

Resistance

Indeed, if China turns out to have ten million or one hundred million people more than previously thought, the leadership will be tempted to use stronger measures to enforce population control. China has been importing food but has little hard currency to buy a lot of it from abroad. So the implication of extra people is ominous. Paradoxically, the successful population control measures of recent years have just been undercut by the state's effort to increase food production. By allowing farmers to keep their produce and turn over a pre-set quota to the state, local authorities have lost a major source of control over households, including the number of children a couple has. At the same time the farmers, given a chance to keep what they produce, have an incentive to have more children for additional labour. So resistance to population control, which is embedded in the Chinese culture, may stiffen just when more babies are the last thing the country needs.

Deborah Shapley

China percentage age distribution 1953-80

Ages (years)	1953	1964	1980 estimate
0-4	15.59	14.52	9.11
5-9	10.94	13.65	12.55
10-14	9.39	12.52	12.93
15-19	9.10	9.01	9.05
20-24	8.24	7.37	8.29
25-29	7.76	7.31	9.06
30-34	6.88	6.77	7.19
35-39	6.41	5.97	5.35
40-44	5.56	5.17	5.02
45-49	5.01	4.47	4.59
50-54	4.24	3.84	4.11
55-59	3.61	3.27	3.59
60-64	2.90	2.56	3.08
65-69	2.03	1.69	2.47
70-74	1.37	1.07	1.80
75-79	0.61	0.55	1.84
80+	0.35	0.26	—

Sources: 1953, Chinese census. 1964, Chinese census. 1980, US Census Bureau estimates based on official Data Model B, May 1980.

CORRESPONDENCE

Nuclear hazard

SIR — While it is true, as N. Bielsten states (*Nature* 25 March, p.284), that thousands of people have died over the past 20 years in dam failures, it is naive to compare this to the record of the nuclear power industry. The fact remains that nuclear power reactors represent the only form of civilian technology with the potential to kill several million people in a single accident.

While one can argue *ad infinitum* about the probability of such an event, we must admit that there have been several near misses and, as more reactors are built and as present reactors age and become more prone to failure, the likelihood of such an accident is increasing. For the nuclear power industry to congratulate itself for the fact that no such accident has yet occurred is reminiscent of the fellow who jumped off the Empire State Building and who was heard to exclaim as he passed each floor "So far, so good".

ROBERT J. YAES

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Fear not frenzy

SIR — Your alliterative headline "Freeze frenzy hits US" in *Nature* of 29 April (p.790) was misleading, and gave a wholly wrong impression about feelings here. There is no frenzy: we are just frightened, and rationally so. I don't want nuclear bombing to destroy me and my family, my home, our laboratories and libraries and universities and so on, but if any major nuclear war starts there seems very little doubt that all this would happen in the first few hours. I imagine that reasonable people would feel similarly in Britain, the Soviet Union and elsewhere. At a recent meeting in here in San Diego, some 3,000 people gathered to discuss nuclear disarmament. The discussions were sober — and sobering — but not frenetic.

RALPH A. LEWIN

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California, USA*

Tangible results

SIR — Martin Raff (*Nature* 25 February, p.642) has observed a disquieting trend towards restricted distribution among scientific colleagues of biological materials.

When research results are in the form of biological material, such as desired gene sequences cloned in a plasmid vector, simple publication of such research results may not be adequate, or simply not most efficient, to advance science.

Such research results should be made available openly and promptly to scientific colleagues, whether in academe or industry. This is the basic premise of the Tangible Research Property policy recently issued at Stanford to affirm appropriate scientific practice (*Nature* 25 March, p.283). The policy specifically adjures against withholding distribution for commercial reasons. Cell

lines, including hybridomas, are distributed to colleagues. Broad distribution of Tangible Research Property (TRP) can be done in a fashion which reasonably protects commercial rights. The optimal mode of protecting TRP would involve strictly limited distribution or secrecy, not options for a university to follow.

With the foregoing as background, let me turn to Dr Raff's concern about open distribution of hybridomas and monoclonal antibodies. There is a great demand for monoclonal antibodies, as is well known to readers of *Nature*. Indeed, this issue of *Nature* may have several advertisements offering various monoclonal antibodies for sale. Producing and distributing monoclonal antibodies to large numbers of users is beyond the capability or funding of the normal university laboratory. Private companies provide a significant service to science by making available well characterized monoclonal antibodies of consistent quality to academic and industrial scientists for their research. As monoclonal antibodies find their way to clinical applications, the role of industry will be of even greater significance.

At the point of clinical application, some measure of proprietary protection for commercial sales is needed by industry to enable its considerable investment to bring therapeutic products to market. Exchange of biological materials or other products should and can be accomplished in a fashion that preserves such proprietary protection and thus avoids jeopardizing the public's access to therapeutic or other products derived from such biological materials. This may involve prior (to distribution) agreement by receiving scientists to follow steps not to destroy those proprietary rights needed for commercial development.

Molecular biology is in transit to an era of increased practical applications for public benefit. The commercialization aspect of this can be depressing, as Dr Raff observes, but the beneficial aspect should not be overlooked.

NIELS J. REIMERS

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Office of Technology Licensing,
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California, USA*

Billions and upwards

SIR — All support to your "ban the billion" campaign — what use is a scientific word that can mean 10^9 or 10^{12} ? Since the ambiguous terms billion, trillion, quadrillion, quintillion, sextillion, septillion, octillion, nonillion (see 6th edition of *Concise Oxford Dictionary*) all end in -llion (and this is therefore a suitable suffix for all large number words) could we not have general agreement on gigillion 10^9 , terallion 10^{12} , petallion 10^{15} , exallion 10^{18} ? As far as I am aware, none of these words has been used before, and certainly not to denote different numbers from those suggested here.

Larger numbers can be constructed by using two (or more) of the internationally recognized prefixes, for example, megaexallion for 10^{24} , exaexallion for 10^{36} . Cubi- will take us even further, cubiexallion (10^{18})³ being 10^{54} .

DAVE E. PARRY

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Hamilton, Scotland, UK*

Boldly going

SIR — Wallis (*Nature* 15 April, p.598) appears to support Hoyle's view that there has been insufficient time for the development of life systems on the Earth. In particular, he says that 2,000 proteins of a specified character are required to catalyse the reactions involved, and that these would not have appeared by random assortment of amino acids into polypeptide chains in the time available.

This argument would be valid if the development of life on planets like the Earth depended upon the simultaneous presence of about 2,000 different polypeptide chains, each containing a uniquely specified group of about 6 amino acids to catalyse a particular biological reaction. This is not true.

The emergence of life did not have to await the appearance of a particular collection of proteins. It emerged from the random set of proteins that were already present on the primitive Earth.

Earth's biochemistry is a by-product of the continuous irradiation of the primitive ocean, producing chains of reactions and processing organic molecular systems back down to the free energy gradient. As has been shown experimentally, irradiated systems of appropriate composition contain all the organic forms (proteins, nucleotides, carbohydrates and so on) required for Earth's biochemistry. The huge variety of peptide chain segments present would provide catalytic centres for many of the reactions involved. The self-replicatory and evolutionary properties of systems of proteins and nucleotides then ensures that the peptide-catalysed chains of reactions play an increasing role in the flow down the free-energy gradient, perhaps by the mechanisms described by Black¹. At some arbitrarily chosen stage in this process of development, we should recognize the system as a form of life.

Wallis's argument does, however, show that no two planets in the Universe can have the same biochemistry. Captain Kirk and the crew of the *Enterprise* are no more likely to find life forms with the Earth's biochemistry than to find them speaking English.

A. E. ROUT

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1. Black, S.: *On the Thermodynamics of Evolution (Perspectives in Biology and Medicine)*, Vol. 21(3), Spring 1978.

PhD job centres

SIR — In his letter published in *Nature* on 13 May (p.98), A. F. W. Coulson describes an information exchange for PhD students in the life sciences and speculates why this has not been done previously in other fields. May I point out that in physics such a compendium was first published by the Institute of Physics in 1969 at the request of the Standing Conference of Professors of Physics. The Sixth Edition has recently appeared.

I too, Sir, have often wondered why similar volumes have not appeared for other subjects.

L. COHEN

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NEWS AND VIEWS

Gravitational wave backgrounds and the early Universe

from B.J. Carr

COULD the detection of a background of gravitational waves tell us what the Universe was like during its first few moments? This is the intriguing question raised in a recent paper by Adams, Hellings, Zimmerman, Farhoosh, Levine and Zeldich in the *Astrophysical Journal* (253, 1-18; 1982). Those interested in detecting gravitational waves have usually focused attention either on bursts of radiation (such as might be generated by supernova explosions or black hole formation) or on continuous radiation (such as might be generated by binary stars and pulsars). Strenuous efforts to detect these types of radiation, using resonant bars and laser interferometers, are already afoot. Attention is now turning to the possibility of detecting a background of gravitational radiation, analogous to that of electromagnetic radiation. Such a background would have to have been generated at a cosmological redshift — that is, well before the present epoch, and perhaps before galaxies formed — so any success in detecting it could give vital information about the early Universe.

One way in which a background of cosmological gravitational waves could arise is from the superposition of many individual bursts generated astrophysically at some time in the past. If the waves were generated early enough (necessarily at redshifts exceeding 1) and if the events producing them were sufficiently numerous, then the gravitational bursts should overlap in the sense that their duration t should exceed their average separation Δt . This would produce a stochastic background with an effective dimensionless amplitude larger than that of the individual bursts by a factor $(t/\Delta t)$. Overlapping bursts could have been generated by various postgalactic events. They could also have been produced by pregalactic events; for example, by the formation of a population of pregalactic black holes. Such holes might today provide the 'missing mass' known to reside in clusters of galaxies and galactic haloes. The period and amplitude of the resulting background will, in this case, have been uniquely determined by the holes' mass and density formation epoch, so its detection could help us ascertain the nature of the missing mass.

Another possibility, and one that is particularly relevant to the Adams *et al.* paper, is that the background could be primordial in the sense that it goes all the way back to the big bang and is part of the initial conditions of the Universe. For example, it is

possible that quantum processes in the early Universe produced a thermal background of gravitons with a present temperature of the order of 1K, analogous to the thermal background of 3K microwave photons which was discovered in 1965. Unfortunately, it seems unlikely that such a thermal background of gravitons could ever be observed. A more interesting possibility is that primordial waves might have resulted from purely classical processes if the early Universe, instead of having the smooth structure observed today, was chaotic. In this case, the waves would not look like radiation at early times because they would have a wavelength larger than the Universe's particle horizon size (the distance travelled by light since the big bang). Also, their dimensionless amplitude, instead of being small, would be of the order of unity and thus severely distort the background space-time. This contrasts to the situation with an astrophysically generated background, where the waves could never have a significant cosmological effect.

There is no doubt that solutions to Einstein's equations which contain incipient gravitational waves in this sense ought to exist, although the complexity of the equations makes the identification of such solutions non-trivial. However, Adams *et al.* have succeeded in providing a formalism with which to describe plane gravitational waves in Bianchi cosmological models. (The Bianchi models are homogeneous in the sense that they look the same everywhere, but anisotropic in the sense that they look different in different directions.) By confining attention to plane-wave solutions, which break the homogeneity of the Bianchi model only in the direction of wave propagation (the z -axis), they manage to find exact solutions which do indeed exhibit the sort of properties anticipated above: at early times the Universe is highly inhomogeneous, with the anisotropy parameter depending on the z -coordinate, while at late times one gets waves travelling in the z -direction. They thus show explicitly how the chaotic behaviour near the initial singularity can be transformed into gravitational waves. Admittedly, the chaotic early Universe is unlikely to possess the plane symmetry assumed by Adams *et al.* A genuine stochastic background, with overlapping waves coming from all directions, would

need to derive from a much more complicated initial behaviour. Nevertheless, it is an important first step and the study of more complicated models will doubtless follow.

An interesting characteristic of gravitational wave backgrounds, whether primordial in origin or generated astrophysically, is that they could have considerably longer periods than the kinds of waves generated by sources in the present epoch. The period of primordial waves is more or less arbitrary but could, in principle, be anything up to the age of the Universe; pregalactic black hole events could generate waves with present periods up to a year. Although such long-period waves could not be detected with conventional bar-type detectors, they could be using more exotic methods. For example, they might be detected by Doppler tracking interplanetary spacecraft: a spacecraft would be jiggled by gravitational radiation and this would be registered as a change in the frequency of the radio waves which are routinely used to track it. Long-period waves could also be detected by their perturbations on planetary orbits. Since the precision with which planetary orbits and spacecraft motions can be monitored is already close to that required to detect possible gravitational wave backgrounds, it is conceivable that gravitational wave astronomy will start in space rather than on Earth.

If waves of primordial origin were discovered, it would obviously be important to ask what information they could yield about the initial state of the Universe. If the early Universe really were as simple as the Adams *et al.* model presumes, that is, inhomogeneous in only one direction, then measurements made by only three spatially separated observers would suffice to infer the initial state. However, the Universe, if chaotic at all, is unlikely to have been that simple and so in practice many more observers would be required to draw any cosmological conclusions. Nevertheless it is intriguing that, in principle, monitoring the positions of planets and satellites could answer questions about the initial singularity. It should be noted that in the Adams *et al.* solutions, the gravitational wave amplitude decreases with time and thus becomes increasingly difficult to detect. Unfortunately, this decrease only occurs on a cosmological time scale, so this feature may not convince organizations which fund gravitational wave research of the urgency of such measurements! □

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Stop-go proteins

from Graham Warren

SOME ten years ago the discovery of the signal sequence^{1,2} showed how, in principle, the cell could 'tag' proteins destined for secretion. When a secretory protein is synthesized the first part of the growing polypeptide chain to emerge from the ribosomal complex is a 'signal' or 'leader' sequence that directs the ribosomal complex to the membrane of the endoplasmic reticulum (ER). There the signal sequence is removed and the rest of the protein synthesized as it is transferred across the membrane into the cisternal space.

Such discoveries were made possible by the development of cell-free systems that mimicked the *in vitro* transfer process. Signal sequences have subsequently been found on all nascent proteins initially inserted into the ER membranes. What has remained a mystery, however, is the way in which the signal sequence carries out its function. Other cellular proteins must be involved but their study requires a painstaking dissection and reconstitution of the ER membrane. Progress in this area has until recently been slow, but now two ER proteins have been characterized^{4,5}: one stops synthesis of the protein until a suitable site for transfer across the ER membrane has been found and one, described in this issue of *Nature* (see p.647), 'docks' the protein at a suitable site.

It has been known for several years that microsomal vesicles (derived from dog pancreatic ER) lose the capacity to transfer secretory proteins *in vitro* when extracted with high salt solutions. Activity can be restored by a component of the salt extract — a protein complex containing six different polypeptide chains⁹ — but the precise function of the protein complex was unknown. Peter Walter and Gunter Blobel have recently shown^{4,5} that it binds strongly to those polysomes synthesizing secretory proteins and stops further translation, but binds only weakly to free ribosomes and has no effect at all on polysomes synthesizing cytoplasmic proteins. Translation of secretory proteins is stopped after the nascent chain has reached a length of about seventy amino acids. Forty of the amino acids will be buried within the large ribosomal complex and thirty will have emerged from the ribosomal complex — the same length as the signal sequence for the secretory protein under study. The signal sequence is rich in leucine which can be replaced in the cell-free system by a more hydrophilic analogue, β -hydroxyleucine. When this is done the protein complex can no longer

effectively inhibit synthesis of the now-modified secretory protein. The authors therefore conclude that the complex recognizes the signal sequence and have termed it the signal recognition protein (SRP). Though much work needs to be done to determine the individual contribution of the six polypeptides to the inhibition of translation, the complex has been found to perform the same function with other secretory proteins⁶ (and see p.647) and at least one membrane protein⁹.

The block in translation can be released by addition of the salt-extracted microsomal vesicles and subsequent synthesis is then coupled to transfer. The ER proteins responsible for releasing this block were unknown but one protein essential for *in vitro* transfer¹⁰ had been previously characterized by Meyer and Dobberstein. It is released from salt-extracted microsomal vesicles by protease treatment and has a molecular weight of 60,000. Antibodies to this proteolytic fragment¹¹ showed it to be derived from an ER membrane protein with a molecular weight of 72,000 that is entirely restricted to the ER *in vivo*. The work reported in this issue of *Nature* (p.647) now shows that the

purified proteolytic fragment specifically releases the translation block induced by SRP. The membrane bound form of the protein has the same effect. This means that the block on synthesis produced by SRP will not normally be released until the SRP has bound to the membrane protein. The protein thus acts to 'dock' the ribosomal complex at a suitable site on the ER.

To accord with the overall scheme for the homing step proposed by Walter and Blobel^{4,5} at least some of the SRP should be free in the cytoplasm ready to bind to polysomes synthesizing proteins initially destined for the ER. SRP was, however, characterized from a salt-extract of microsomal vesicles. The cytoplasmic form has now been detected (see p.647) using a sensitive assay and significant amounts of SRP have been found in the cytoplasm of reticulocytes and dog pancreatic cells.

The homing step explains how the ribosomal complex becomes specifically bound to the ER membrane. The signal sequence does not play a direct role in this step but acts through SRP. This may have an additional advantage since the hydrophobic signal sequence would not be free to interact with any cellular membranes other than the ER. Once it is free to interact with the lipid bilayer, the homing step will have ensured that this bilayer will be that of the ER. The inhibition of translation is clearly an important part of SRP's function; to

SPEAKING on 27 May at a Royal Society discussion meeting on Cell Membranes and Glycoprotein Synthesis, Dr Günter Blobel of the Rockefeller University gave advance notice of a new and unexpected twist in the long running saga of the signal sequence. It now seems that the signal recognition protein, whose role is described in the accompanying article, has a specific ribonucleic acid associated with it.

That the ribonucleic acid plays some, as yet undefined, part in the process of ensuring that secretory proteins are secreted is suggested by the inhibitory action of ribonuclease on the ability of the signal recognition protein to arrest the translation of secretory proteins (see accompanying article). The sedimentation coefficient and partial sequence of the ribonucleic acid associated with the signal recognition protein make it all-but certainly identical to 7S RNA, the sequence of which has just been described by two independent laboratories (E. Ullu, S. Murphy & M. Melli *Cell* 29, 195; 1982 and W. Li, R. Reddy, D. Henning, P. Epstein & H. Busch *J. biol. Chem.* in the press). Intriguingly, 7S RNA is closely related to the 'Alu' family of RNAs, the genes for which exist in many thousands of copies scattered throughout the mammalian genome. Alu RNAs, although present in abundance in most

cells, are of unknown function. It could be that the role of one subset has now been unearthed.

At the same meeting Blobel acknowledged the demise of a different twist in signal sequences. His classic signal sequence is at the amino terminus of a secreted protein and is cleaved off as the protein traverses the membrane. Later it emerged that ovalbumin, although a secreted protein that competed with others for insertion into microsomes *in vitro*, did not subsequently lose an amino-terminal peptide. In 1979 Blobel's laboratory published a paper (V.R. Lingappa, J.R. Lingappa & G. Blobel *Nature* 281, 117) claiming that the signal peptide of ovalbumin was internal, and suggesting a location around amino acid 250 of the 385-residue chain. Now, as Blobel acknowledged, it looks as if that result was in error. One is instead to believe the conclusion of William Braell and Harvey Lodish (*J. biol. Chem.* 257, 4578; 1982) that the signal sequence of ovalbumin is on the amino-terminal side of residue 150 and thus not exceptional.

But, according to an earlier paper of Braell and Lodish (*Cell* 28, 233; 1982), what is not true for ovalbumin is true for the erythrocyte anion transport protein which really does have an internal signal sequence somewhere around its middle.

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stop translation must be a complicated process reflected in the polypeptide complexity of SRP.

The advantage of stopping translation until a site on the ER has been found is not hard to see. Without this block translation would continue and the ribosomal complex would only have a few seconds to reach a suitable site for transfer of the protein. If it did not reach a suitable site in time the protein would be completed and discharged into the cell cytoplasm. This could be very damaging particularly if, for example, these proteins were degradative enzymes. By stopping translation SRP make sure that there is sufficient time to find a suitable site.

These arguments may not apply in prokaryotes which are much smaller (so that less time would be needed to reach a membrane) and have only one membrane (the plasma membrane) through which proteins are initially transferred. It is even not clear that proteins must be transferred during synthesis¹³ and in one case (M13 phage coat protein) transfer can occur after synthesis and apparently requires only a suitable lipid bilayer and signal peptidase^{14,15}. It would therefore be important to see whether prokaryotes have the equivalent of SRP and a docking protein since their presence would argue strongly in favour of transfer during synthesis.

The homing step is distinct from, and precedes, the transfer of the nascent protein across the ER bilayer. The ribophorins and the signal peptidase³ may be involved in this step but no other cellular proteins have been characterized. Reconstitution techniques now need to be applied to the transfer step. It will be more difficult to study because the ER lipid bilayer will have to be broken down and built up again. Nevertheless, the striking success of reconstitution techniques in revealing the functions of signal recognition protein and docking protein make the attempt well worthwhile. □

Related genes can have unrelated introns

from Athel Cornish-Bowden

IN eukaryotic gene families, such as those that code for the globins, the number and position of the introns has been found to be conserved across each family and, presumably, during evolution. A very different picture emerges from new work reported in this issue of *Nature* (p.655) by Woo and his collaborators. They have studied the introns in the genes for two distantly related proteins, human α_1 -antitrypsin and chicken ovalbumin, and have found that there is no correspondence between the three introns of the ovalbumin gene and the seven of the α_1 -antitrypsin gene. This result calls into question the idea that the arrangement of introns provides a record of the original structure of a eukaryotic gene; instead it suggests that introns may have been introduced into genes since the divergence of the vertebrates.

Ovalbumin is the principal protein in chicken egg white, whereas α_1 -antitrypsin is a human plasma protein involved in the control of elastase; individuals deficient in it have a high risk of lung disease. There was no reason to expect any sequence similarity between the two proteins and the observation that they are in fact 24 per cent identical was thus a surprise. The degree of similarity is far too high to be ascribed to chance, as Hunt and Dayhoff (*Biochem. biophys. Res. Commun.* **95**, 864; 1980) estimated that such a possibility would occur with a probability around 10^{-41} .

If chance is excluded, there are two ways in which the similarity between ovalbumin and α_1 -antitrypsin can be explained; either the two proteins have diverged from a common ancestral protein, estimated by Hunt and Dayhoff to have existed about 500 million years ago; or they have converged to similar sequences as a consequence of selection. In the former case, the lack of correspondence between the arrangements of introns in the two genes needs to be explained; in the latter it does not because no correspondence would be expected. Woo and collaborators say that it is impossible to determine conclusively whether the two proteins arose by convergent or divergent evolution. I believe them to be unduly cautious in this regard and that the possibility of convergent evolution can be discounted.

Although examples of convergent evolution of protein structures do exist,

they are observed at the level of catalytic mechanism and not in amino acid sequence. In the best established case, that of the bacterial proteinase subtilisin and the mammalian serine proteinases such as chymotrypsin, the catalytic mechanisms are essentially the same but there is no sequence similarity beyond a use of the same kinds of residue in the catalytic action. By contrast, if the similarity between ovalbumin and α_1 -antitrypsin arose by convergent evolution it would imply convergence to a high degree of similarity over the whole lengths of two proteins without even, apparently, a similar physiological function. If this were substantiated its shattering effect on current ideas about protein structure and evolution could hardly be exaggerated; it would be comparable with the effect of the first reports of introns in eukaryotic genes and overlapping genes in viruses on our understanding of gene structure.

The chicken ovalbumin gene contains seven introns, all of them located in the 5' half of the mRNA, whereas the three introns in the human α_1 -antitrypsin gene are all in the 3' half of the mRNA. Only one intron in the ovalbumin gene occurs in even approximately the same position as any intron in the α_1 -antitrypsin gene: the introns in question do not show significant sequence similarity either with one another or with the corresponding coding regions of the other genes. It seems inescapable that a substantial reorganization of the intron structure has occurred since the time of the common ancestral gene shared by the ovalbumin and α_1 -antitrypsin genes.

Could the differences in intron structure be a consequence of random losses of introns during evolution from an ancestral gene containing at least ten introns? If there were exactly ten, of which a random three were deleted on the lineage leading to the ovalbumin gene, and a random seven were deleted on the lineage leading to the α_1 -antitrypsin, the probability that the two descendant genes would have no introns in common would be $3!/10!$, or $1/120$. This probability is hardly small enough to rule out random deletion of introns as an explanation, but it is small enough to suggest that alternative explanations should be considered, not only in this case but also in that of the actin gene family; for which a much smaller degree of intron variability has been observed. Woo and collaborators suggest that some at least of the introns may have been introduced into the ovalbumin and α_1 -antitrypsin genes after they diverged in evolution. If so, the arrangement of introns must be a more

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recent aspect of gene structure than has been supposed. Light will perhaps be shed on this question by investigation of the intron structure of the gene for human

antithrombin III, another plasma proteinase inhibitor in the super-family that contains ovalbumin and α_1 -antitrypsin. □

Membrane kissing

from A. W. Cuthbert

CELLS that respond very rapidly to chemical signals such as neurotransmitters change their ionic permeability by altering the functional state of ion channels already present in the membrane. An alternative but improbable way of changing permeability might be to insert patent ion channels into the membrane from a preformed store within the cell. However for cells that respond only slowly to signals, as with some hormones, such an alternative way of changing permeability could have advantages. For instance, a sustained response might follow only a transient exposure to hormone with return to the prestimulated condition being governed by membrane turnover processes.

Lewis and de Moura report in this issue of *Nature* (p.685) what may be a convenient way to study the insertion of ion channels into cellular membranes from an intracellular source. The tissue they have exploited is rabbit urinary bladder epithelium, which transports sodium electrogenically from the apical to the basolateral side. They "punched" stripped preparations of this tissue by subjecting the apical side to a series of rapid changes in hydrostatic pressure. As a result, transepithelial resistance was reduced and short-circuit current increased. Punching caused more than a tenfold increase in current together with a significant increase in the sodium selectivity of the apical face. The current, like the normal sodium transport, was sensitive to the pyrazine diuretic amiloride. The authors suggest that punching causes cytoplasmic vesicles containing sodium channels to collide and kiss the apical membrane, resulting in the transfer of sodium channels to the apical surface.

The urinary bladder, of course, undergoes cyclical changes in volume as it collects urine. Lewis and de Moura also show that when the bladder is distended mechanically the membrane capacitance remains rather constant while the mass per unit area is decreased. This, they argue, is due to the insertion of new membrane material from an intracellular source keeping the capacitance constant at around $1 \mu\text{F cm}^{-2}$. Similarly, when the cells are allowed to swell in a hypotonic medium

there is an increase in apical membrane area which results in a 74 per cent increase in apical membrane capacitance. The effects both of punching and of exposure to hypotonic solutions were blocked by cytochalasin B, perhaps indicating the involvement of microfilaments.

An unexplained finding was that although punching increased apical sodium permeability there was no increase in capacitance. If vesicles rich in sodium channels are incorporated into the apical membrane when it is punched, then equivalent amounts of membrane with fewer sodium channels must be removed on rebound.

Undoubtedly these experiments will give investigators another way to manipulate the apical membrane of transporting epithelia. As with many discoveries, their importance is not necessarily that they are new but rather that they are timely. Insertion of channels into epithelial membranes as a way to increase permeability has been proposed for both sodium and water in recent years. As far back as 1968, Nutbourne¹ applied small hydrostatic gradients to the apical surface of frog skin and recorded marked increases in sodium transport, yet this attracted little attention although the technique used was very similar to that described by Lewis and de Moura. It is interesting to re-read the discussion of that paper written at a time when the mechanisms for sodium ion translocation across the apical face were poorly understood.

The mammalian urinary bladder, like numerous other tight sodium-transporting epithelia, responds to aldosterone with an increase in ion transport. Early studies^{2,3} and more recent electrophysiological measurements⁴ have shown that the sodium permeability of the apical surface of epithelia increases under the influence of this mineralocorticoid. Current fluctuation analysis in potassium-depolarized toad bladders has shown that the unit conductance and average lifetime of individual sodium channels are not changed but that there is an increase in the number of electrically active sodium channels⁵. Although the action of aldosterone requires DNA-dependent RNA synthesis and protein synthesis it does not follow that aldosterone actually causes the synthesis of new ion channels. The metabolism of the cell may be altered in such a way that preformed ion channels

stored in cytoplasmic vesicles are inserted into the apical membrane, without ruling out an additional effect on synthesis. Recruitment of electrically silent channels seems also to be responsible for the change in the sodium permeability of the apical face in amphibian urinary bladders treated with antidiuretic hormone (ADH)⁶, but whether this occurs from electrically silent channels in the apical membrane or by fusion of cytoplasmic vesicles is not known. True, there is an increase in the membrane capacitance in toad bladder in response to ADH⁷ which might be expected from the insertion of new membrane. However, there are two problems with this interpretation. First, Lewis and de Moura did not see an increase in capacitance in their punched tissues although sodium transport was markedly increased. Second, ADH also causes an increase in water permeability of the toad urinary bladder which may be responsible for the capacitance change.

Painstaking morphological studies using freeze-etching have shown that ADH causes the appearance of particle aggregates in the cytoplasmic face of the apical membrane^{8,9}. There is strong correlative evidence that the particle aggregates are associated with increased water permeability, but whether the aggregates are formed by translational movement in the membrane or by insertion from the cytoplasm is not clear, although the increase in capacitance favours the latter view. Interestingly, methohexital blocks the water permeability response to ADH without affecting the sodium transport response¹⁰. Whether the whole of the capacitance increase is also blocked by methohexital remains to be explored.

Membrane macromolecules must be made within the cell so it is not unlikely that

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molecules *en route* for the membrane, and in increasingly complete stages of assembly, will be found in the endoplasmic reticulum, Golgi and cytoplasmic vesicles. Reservoirs of a number of molecules destined for the cell membrane and showing modified immunoreactivity, enzymatic potential or binding properties have been identified intracellularly¹¹⁻¹⁵. A recent example, relevant to this discussion, is the identification of a saxitoxin-binding protein in frog heart cytosol as a putative sodium channel¹⁶. Amiloride analogues have been used to identify components in sodium-transporting epithelia with properties consistent with those of apical sodium channels. Binding measurements in intact tissues, such as frog skin¹⁷, have given site densities (130 μm^{-2}) comparable

with fluctuation analysis¹⁸ (50 μm^{-2}). However, in isolated cells¹⁹ and homogenates²⁰ where the ligands have access to intracellular sites, much greater site densities have been obtained. Finally, in the epithelium lining the avian coprodaeum, rod-shaped particles, tentatively identified as sodium channels, have been seen in freeze-fractured membranes, both apical and intracellular²¹.

Thus there is a body of very different sorts of evidence which suggests that transporting epithelia have a reservoir of vesicles containing sodium channels poised for insertion into the apical membrane. Whether these vesicles can 'kiss and make up' the increase in sodium permeability of the apical surface in response to punching may be very hard to prove. □

below ground¹⁶. Ground beetles (*Carabidae*), common in many habitats^{17,18}, store seeds in the lining of their tunnels. The function of this behaviour is obscure, but the seeds appear to remain unharmed^{19,21}.

Earthworm burrows and casts are known to be richer in critical plant nutrients than surrounding soils^{5,22} and many kinds of seeds are passed intact and viable embedded into the cast²³. Snails too may be of importance. A series of experiments in which seeds were placed with captive snails showed that the overwhelming majority pass through the digestive tract to be deposited in the faeces intact.

We suggest 'inhumation' (Latin: *humare* = to cover with earth) may provide a useful term for the process described above. While this process has been described for seeds passing through the guts of vertebrates such as birds, bats and various other mammals²⁴⁻²⁷, the inclusion of major and abundant invertebrate groups, such as ants, beetles, earthworms and snails, may make the process of inhumation far more important than previously supposed. Mortality in plant populations is generally greatest among seedlings deprived of suitable microsites or sufficient seed reserves. Inhumation may circumvent these dangers to some degree by increasing the probability that seeds are relocated to microsites already provisioned with nutrients. □

Inhumation: how ants and other invertebrates help seeds

from Andrew J. Beattie and David C. Culver

WHAT happens to seeds once they come to rest following dispersal? Seeds are scattered widely in the environment by wind, water, animals and explosive mechanisms and yet the sites where successful germination and growth can occur are often patchy, scarce and limiting^{1,2}. Some possess mechanisms such as hygroscopic bristles for self-burial³, while others are effectively buried by falling into soil crevices or where wind- or water-borne soil and debris accumulate². Many seeds do not require burial for germination, but a common fate for those remaining on an exposed surface is to be eaten⁴. Are there mechanisms that increase the probability with which they will end up in a better site?

Recent work on ant dispersal of seeds (myrmecochory) strongly suggests that a major selective advantage is provided by the relocation of seeds to ant nests which are richer than surrounding soils in essential nutrients such as phosphorus, potassium and nitrogen⁵⁻⁷. Seeds end up on the soil or litter surface following dispersal and are then removed by ants which are attracted to nutritious tissues (elaiosomes) on the seed coat. Once in the nest, the elaiosomes are removed and the seeds abandoned, intact and viable, either in an old gallery or in the refuse pile⁸.

In a series of experiments with two myrmecochorous species of *Viola*, the fates of seeds taken into nests by ants were compared with the fates of seeds planted at random in the same habitats and it was found that seedling emergence is nearly

three times more likely from nests. Furthermore, the seedlings emerging from ant nests are larger, and the probability of their surviving the first two years is considerably higher (0.14 against 0.03)^{7,9}.

Additional ant-mediated effects appear in a study of another myrmecochore, *Sanguinaria canadensis*. This species can persist in disturbed habitats, where the primary ant dispersers are absent, by means of vegetative propagation but, in undisturbed forests, where seed-dispersing ants are present, profound changes in adult density, dispersion and patterns of reproduction are brought about¹⁰. Further beneficial effects also come from the greater chance that seeds relocated to nests will escape the attention of predators^{4,11}.

Myrmecochory may be much more widespread than has been thought. European and North American temperate deciduous forests and Australian sclerophyll shrublands have often been regarded as its lone strongholds^{12,13}, but we now know that it occurs commonly in a very wide variety of plant community types worldwide, from low to high latitudes and elevations in both the Northern and Southern Hemispheres⁹. Myrmecochorous species are known from 80 plant families (including grasses and cacti) and may be trees, shrubs, vines, herbaceous perennials, annuals, hemiparasites, parasites and epiphytes. Myrmecochores constitute 35 per cent of species in certain habitats, and up to 76 per cent of emergent stems¹³⁻¹⁵.

Invertebrate groups other than ants may have similar effects on the fate of seeds. Scarab beetles (*Scarabaeidae*) in the Cerrado of Brazil collaborate to roll and bury fruits. The larvae mature by eating the pulp and the viable, intact seeds remain

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A beginner's guide to major histocompatibility complex function

from Polly Matzinger and Rose Zamoyska

The glycoproteins encoded by the major histocompatibility complex (MHC) are guides by which cells of the immune system distinguish both each other from all other tissues, and also all cells of the body from non-cells (such as bacteria, viruses, toxins, etc.). These two different modes of discrimination require two different sets of MHC-coded cell surface molecules, Class I and Class II.

Class I molecules are characteristic of cells

An example of the importance of the recognition of cells versus non-cellular material can be seen in the immune response to a virus infection, which is fought mainly by a subset of T lymphocytes called T killer cells. This set of lethally destructive lymphocytes can kill virus infected cells before synthesis of new viruses is complete, thus preventing the spread of the infection. But how do T killer cells distinguish between *virus infected cells*, which they can destroy, and free virus particles, which they are powerless to stop?

The T cell population consists of millions of constantly circulating lymphocytes, each one bearing receptors able to bind to one particular three-dimensional structure. This results in a heterogeneous population which can recognize a wide variety of foreign molecules. Normally these T cells are quiescent and must be activated to become effective killer cells. Their activation is not a simple process but requires the participation of a special accessory cell called the "antigen-presenting cell". A virus entering the body is picked up by the presenting cells which process it and present it to the roving T cell population. Those few T cells which have receptors specific for viral structure will bind to the presenting cell. Although there may be resting T cells which can bind to virus alone, these are not activated. Only those T cells which recognize *both* the Class I cell surface molecules and the viral structures on the presenting cell are induced to proliferate and differentiate into active killer cells. Since induced T cells maintain their specificity, and since only those T cells which bind both viruses and Class I cell surface molecules are induced, the resulting activated killer cells are committed to the recognition of cell-bound virus. Furthermore, activated killer cells are extremely precise in their recognition of both virus and MHC molecules, discriminating between closely related strains of virus and also distinguishing between different Class I alleles. An animal bearing a particular Class I allele 'a', for example, will produce killer cells which lyse infected target cells expressing 'a' but will ignore infected target cells bearing 'b' alleles. This obligate recognition of foreign molecules in the context of MHC proteins is called

'MHC restriction' of T-cell recognition.

Nearly all tissues express Class I molecules and, if infected, can be destroyed by active T killer cells. However, in many species, Class I molecules are not expressed by mature red blood cells (having no DNA, red cells cannot support virus replication) nor by sperm (although they are foreign invaders, sperm are usually welcome).

Class II molecules are characteristic of cells of the immune system

While T killer cells cope with cell-bound virus, antibodies secreted by activated 'B' cells deal with free virus particles. In order to be activated, resting B cells require signals from a subset of T cells called (not surprisingly) T helper cells. If a T helper cell specific for virus is to help a B cell make antibody against the virus, it must have the capacity to recognize not only the virus but also the B cell. It would be futile for T helper cells to send signals to cells outside the immune system (a virally infected liver cell, for example) so, unlike T killer cells, T helper cells do not recognize virus in the context of the ubiquitously expressed Class I molecules. Instead they are limited to the recognition of virus in the context of Class II molecules, which are expressed mainly by B cells and antigen-presenting cells. The activation of T helper cells may be described in a similar way to the MHC-restricted activation of T killer cells. Circulating T helper cells encounter virus structures on the surface of a presenting cell. They are activated as a result of interacting with virus and Class II molecules on the presenting surfaces, and will subsequently assist virus-specific B cells carrying the same Class II molecules.

Class II genes were originally discovered as immune response (IR) genes, so called because animals which carried different MHC alleles differed in their ability to make immune responses to certain foreign molecules. This now appears to be explained by the 'guidance' function of the MHC molecules. It is known that a foreign molecule may be recognized more efficiently with one Class II allele than with another. Animals bearing class II alleles that are poorly recognized in association with the foreign molecule will not generate activated T helper cells and will consequently be unable to produce strong antibody responses. It has been suggested that an MHC molecule might be inefficient at presenting a particular antigen because the two molecules do not form a good

complex on the surface of the presenting cell: or, alternatively, that a complex does form, but for various reasons there are no helper T cells able to recognize it.

How do MHC molecules activate T cells?

There are at least two possible ways in which MHC proteins could act as T-cell activators.

1. Like hormones, they might work directly, activating any T cell which binds them.

2. The binding of MHC molecules by T cells may trigger the antigen-presenting cell to send activation signals to the bound T cell. T cells binding virus alone would therefore not trigger the antigen-presenting cell and would not receive the activation signal. The fact that both Class I and Class II molecules are transmembrane proteins, and the observation that not every cell expressing these molecules can act as T-cell inducers, support the latter possibility. Further evidence that MHC molecules act as presenting cell triggers comes from transplantation experiments.

MHC genes, being highly polymorphic, are the major barriers to tissue transplantation. T cells distinguish between the products of self and foreign MHC alleles with great precision, destroying any tissue bearing foreign MHC products. The magnitude of this reaction is greatly reduced if the foreign tissue is first cleared of its resident presenting cells, even though the host's presenting cells should be able to pick up the foreign MHC molecules and activate T cells against them. Thus there is clearly a difference between a foreign MHC molecule in its original presenting cell membrane and the same molecule when it is picked up and displayed by the host's presenting cell. Perhaps the represented foreign MHC is no longer appropriately linked up inside the cell to act as a triggering device. It would then be treated like any other foreign molecule, activating only those T cells which recognize it in association with the host's own MHC proteins. Since the grafted tissue does not express these host MHC molecules, it would not be recognized by the activated T cells and would not be rejected.

What is it about their structure that allows MHC molecules to associate with so many foreign molecules? How is their structure related to their ability to act as triggering devices? Are T cells evolutionarily pre-programmed to look at them or are the specific T cells selected from a randomly generated population of resting cells? How many different MHC molecules are there? With the molecules and their genes in hand (see the following article) we may soon have the answers to these questions. □

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The evolutionary past of the major histocompatibility complex and the future of cellular immunology

from Miranda Robertson

THE penetration of the major histocompatibility complex (MHC) with cloned DNA probes has opened up a new approach to an old evolutionary problem — how to account for the unparalleled polymorphism of the cell-surface antigens it encodes. This spring, representatives of the main laboratories involved met in Oxford to compare notes, and to discuss their strategies for research on the functions of the MHC products.*

It is now generally accepted that in the remote evolutionary past, three of the four most prominent families of proteins of the immune system must have evolved from a single immunoglobulin-like domain. The largest and most complex of the three related families — that of the immunoglobulin genes — is now scattered over three different chromosomes; the other two — the so-called class I and class II MHC antigens — are encoded together in the major histocompatibility complex (Fig. 1), along with some of the fourth family — the components of complement, which so far seem to be structurally unrelated to any of the others. The sequences of cloned MHC DNA have strongly reinforced the evidence for the common ancestry of the immunoglobulins and the class I and class II antigens; this

present state of knowledge on the structure of the MHC genes, their products and their relationships with immunoglobulin genes are summarized in Fig. 2. But most of the discussion at Oxford focussed on the much more recent evolutionary history of the MHC, and in particular on the polymorphism of the class I and class II antigens.

According to genetic and biochemical analysis, there are 30–60 alleles at each of the polymorphic loci encoding the class I antigens, and perhaps 12–40 at each of the class II loci. Why and how has this extraordinary polymorphism been maintained in mammalian evolution? An answer began to emerge with the relatively recent discovery that the MHC antigens guide and regulate the immune response (see legend to Fig. 1). It is known that the T lymphocytes that kill virus-infected cells, as well as those that regulate the responses of other lymphocytes to antigens, can recognize foreign antigens only in

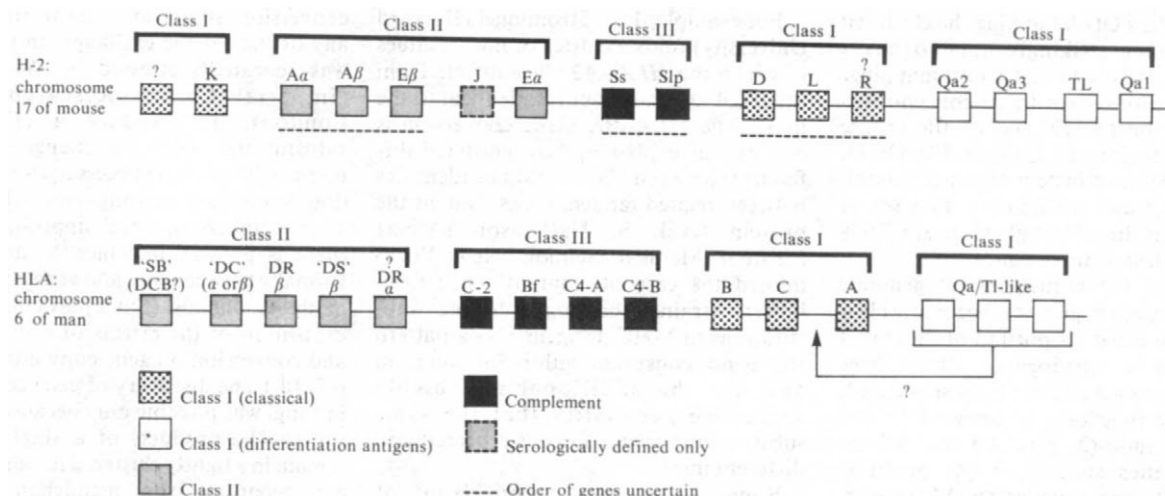
association with MHC molecules (see preceding article). It has also been possible to show that a given foreign antigen may be more efficiently recognized in association with some MHC alleles than with others. Thus an animal heterozygous for MHC antigens may respond efficiently to a wider range of pathogens than a homozygote, and polymorphism may be maintained by heterozygous advantage. The polymorphism may be stable, or the MHC molecules may have to evolve rapidly to keep pace with pathogens that are continually evolving ways to evade recognition, so that advantageous new variants are continually selected. A deeper understanding of the selective pressures at work on MHC molecules will require a much better appreciation of the mechanisms of recognition and destruction of pathogens; and this is likely to be the reward of one of the new techniques discussed at Oxford. For it is now possible to transform cultured cells with cloned MHC genes; and the use of manipulated genes in such experiments may settle these

*A meeting on the 'Cloning of the HLA and H-2 Regions', organized by W. F. Bodmer and sponsored by the Imperial Cancer Research Fund and the European Molecular Biology Organization, was held in Oxford on March 21–24 1982.

Miranda Robertson is associate editor of Nature.

Fig 1 The major histocompatibility complex (MHC) of mouse and man. The MHC molecules can be divided into three classes on the basis of their structure and function. The class I antigens constitute a single class structurally (see Fig. 2a), but fall into two functional groups. The first of these contains the "classical" class I antigens, first discovered as the transplantation antigens and now known to function as target antigens in the recognition and destruction of virus-infected cells by cytotoxic T lymphocytes. They are expressed on virtually all somatic cells. The second group of class I antigens, loosely defined as lymphocyte differentiation antigens because of their tissue distribution, have no known function. The class II antigens are expressed largely on B lymphocytes and macrophages of the immune system, and are believed to be essential for presenting antigen to the helper and suppressor T cells that regulate the immune response. The class III products are components of the complement system. These maps are based on serological and biochemical data: it is now known that the MHC contains other class I sequences not detectable serologically. Moreover, not all mice express all the genes indicated on the *H-2* map: some strains are known to lack a functional *L* gene, and the evidence for a second *K* gene is extremely indirect¹. The *J* gene is the most problematic of all because its product has eluded all attempts at biochemical identification. Because its determinants have been detected on candidate T-cell receptor molecules and antigen-specific factors, the *J* product has become something of an immunological holy grail. Now that Steinmetz has a probe for the neighbouring *Ea* gene (see text), he proposes to walk down the chromosome to the *J* locus: this should settle the question of whether there is anything there (disputed by Klein *et al.*²). The exact distance he will have to walk is not certain.

In the mouse, different groups of alleles have been segregated into inbred strains, each linked group (haplotype) being designated by a superscript letter. Thus B.10 strain mice, for example, have the *b* haplotype, and so bear antigens H-2K^b and so on.



and other fundamental questions that have resisted the efforts of cellular immunologists for many years.

But in the meantime, sequence analysis of the cloned DNA more directly addresses the question of how — rather than why — the polymorphism has arisen. Immunogeneticists have speculated, for example, that the alleles may not be alleles of a single gene at all, but tightly linked clusters of different genes^{7,8}. This is now ruled out. The class I genes do occur in clusters of related sequences, at least in mouse⁹; but although there are at least twice as many class I sequences as would be predicted from genetic analysis, there are not enough to account even for a substantial fraction of the serologically detectable alleles. The extra class I sequences thus pose a fresh evolutionary problem — particularly in the light of preliminary evidence that the class II antigens, by contrast, have few or no supernumerary relatives in the genome. The nature and significance of the extended class I gene family was one of the main topics of discussion at Oxford.

Evolution of the class I genes

The class I genes seem to constitute a large and complex family is a state of evolutionary flux. In all genomes for which DNA probes are available, there are at least twice as many sequences hybridizing with class I probes as there are class I genes on the genetic map. Pigs (D. Singer, US National Institutes of Health, Bethesda) and men¹⁰ probably have 15–20, and mice have as many or more, depending on strain. The largest and most complex class I family discovered so far is that of the BALB/c mouse, which has 36 hybridizing sequences organized in 13 clusters⁹; the B.10 mouse, by contrast, may have only 15–20 (R. Flavell, National Institute for Medical Research, London), and comparisons between restriction maps from other strains reveal yet more variation⁹.

This evidence for a history of recent duplications and deletions is strongly reinforced by two features of the sequence clusters: first, more homologous genes seem to be clustered together; and second, the noncoding DNA flanking the clustered genes is often strikingly homologous¹¹. According mainly to restriction mapping, the duplications seem to be concentrated largely at the *Qa/TL* end of the mouse MHC (M. Steinmetz, Caltech; Flavell; D. Schulze, Albert Einstein Medical School, New York); and preliminary analyses of human cell lines¹⁰ suggest a similarly skewed distribution in man.

Now that the sequencing of genomic class I DNA has begun, it is becoming clear that a substantial proportion of the class I family may be pseudogenes. Of the three BALB/c genes that have been sequenced, two encode functional *L^d* antigens^{12–14} and one is a pseudo-*Qa* gene. Of four human class I genes sequenced (or partially sequenced), one encodes an A2 antigen,

one a B7, one is clearly a B pseudogene and the fourth may be another pseudogene (A. Biro, Yale University, and ref. 15).

Why should so many cousins of the class I genes — some evidently nonfunctional — have been maintained in the mammalian genome if they do not contribute to MHC antigen polymorphism? The answer favoured by most of those working on them is that in fact they do, but indirectly. W. Bodmer (Imperial Cancer Research Fund, London) has speculated that variant proteins might be produced by differential splicing of primary transcripts containing more than one gene. There is so far no evidence for this. On an evolutionary timescale, L. Hood (Caltech) has pointed out that polymorphism could be increased by the tendency of partially homologous sequences to recombine, in particular by unequal crossing-over⁹. Others (P. Kourilsky, Pasteur Institute, and refs 12 and 16) propose, more specifically, that the genes and pseudogenes at the *Qa* and *TL* regions of the mouse may represent 'reservoirs of polymorphic variation' that can be tapped by means of gene conversion. Since the nature and implications of the evidence for gene conversion attracted more discussion than any other speculation on class I gene evolution, it is worth recapitulating briefly how it is believed to work.

Gene conversion, originally found in fungi, is a mechanism by which genetic information can be transferred from one gene to another related gene anywhere in the genome — although allelic genes and members of tandem families are the most likely recipients. It is believed to occur through pairing between partly homologous sequences during meiosis or mitosis, followed by mismatch repair resulting in the conversion of one sequence to the other. There have recently been increasing indications of such cross-talk between different members of gene families in the course of vertebrate evolution; the evidence for gene conversion in the MHC rests chiefly on the discovery of clusters of substitutions, detected both at the protein and at the DNA level, in individual genes.

For example, J. L. Strominger (Harvard University) finds a cluster of nine residues in which the *HLA-A2* allele differs from the *HLA-A28* allele but is identical to the non-allelic *HLA-B7*. Gene conversion is one way of explaining how clustered differences between alleles could be identities between related tandem genes. Still at the protein level, S. Nathenson (Albert Einstein Medical School, New York) quoted the case of 'mutant' mice from inbred strains showing multiple substitutions in MHC antigens¹⁷ — a pattern that is not consistent with point mutation and has the additional and highly suggestive peculiarity that the same substitutions seem to occur repeatedly in different mice.

Some preliminary comparisons of

mutant protein sequences and recently cloned genes now strongly suggest that the mutant sequences derive from other genes. D. Margulies (US National Institutes of Health, Bethesda), for example, comparing a cloned *H-2L^d* gene from a BALB/c mouse with the *H-2K^b* antigen of the B.10 mouse, and with mutant *H-2K* antigens, finds that in 9 out of 10 cases, differences between wild-type and mutant *K^b* sequences are identities between the mutant and *H-2L* sequences. Further eccentric relationships between *H-2* loci are revealed by a comparison of the same *H-2L* gene with other class I sequences: they show that the BALB/c *H-2L^d* gene is more closely related to the *H-2D^b* gene of the C57BL/6 mouse than to other class I genes of its own strain¹². There are two possible explanations for this. The first is that an exchange of information took place between an *H-2L^d* gene and an *H-2D^b* gene before the development of the inbred strains that segregated all the *H-2^d* genes into BALB/c and all the *H-2^b* genes into C57BL/6. Alternatively, the *H-2L^d* gene may have been converted by an unexpressed pseudogene within the BALB/c MHC.

In the light of these possibilities, both Margulies and Flavell are focusing their attention particularly on an *H-2K* mutant, *H-2K^{bml}*, that arose within the B.6 mouse strain. *H-2^{bml}* contains two amino acid substitutions that entail at least four base changes (so that point mutation is unlikely), and are homologous to the BALB/c *H-2L^d* gene. Because the mutation occurred within the B.6 strain, it cannot be due to conversion by an *H-2^d* chromosome, and may be due to conversion by an *L^b* gene or an *L*-like pseudogene in the B.6 chromosome. This hypothesis can be checked by searching the B.6 MHC with the appropriate probe.

Gene conversion is in fact also an alternative explanation for the strong homologies seen in the flanking regions and introns of MHC genes, since conversion would be expected indiscriminately to homogenize coding and noncoding DNA. Indeed, the difficulty of distinguishing between crossing-over and conversion as the mechanism underlying any of the genetic exchanges in the MHC was repeatedly stressed by W. Bodmer (Imperial Cancer Research Fund, London). To produce a cluster of substitutions with no change in gene number it would be necessary to postulate double unequal crossing-over, which may seem cumbersome and improbable; but there is no way in which to distinguish formally between that and gene conversion in higher animals. (For a particularly lucid exposition of the effects of crossing-over and conversion on gene copy number, see ref. 18.) The discovery of gene conversion in fungi was possible only because in some species the products of a single meiosis remain in a tightly clustered tetrad in which any departure from mendelian ratios is

directly detectable. It was the occasional observation of tetrads in which the expected 2:2 ratio for a given allele proved to be 3:1 that revealed the occurrence of gene conversion between alleles.

Such precise analysis is not possible in mice, in which each offspring in a litter is probably the product of a quite different meiosis, and the mendelian ratio for a given litter cannot be expected to be exact. The stability of mendelian ratios averaged over large numbers is, however, notorious, which shows that gene conversion must be rare, and raises a further problem, also emphasized by Bodmer. While various mechanisms can account for the creation of pseudogenes and the conversion of functional ones, it may be much more difficult to account for the spread and fixation of these rare genes in the population. Selection does not anticipate, so the potential advantage of pseudogenes as a bank of variants could not drive a non-functional gene to fixation. There are various general ways of explaining the fixation of genes in the absence of selective pressure: one is through the founder effect, in which a large population grows from a few individuals derived from a rare mutant; another is the hitch-hiker effect, in which the event that creates a nonfunctional gene or a mutation also produces an effect in a closely linked locus that confers an immediate selective advantage. The non-mendelian mode of inheritance of converted genes may be very important in this connection, since it could accelerate spread provided that conversion were more probable in one direction than in the other.

But a more specific, and much more controversial possibility was raised by G. Jay (US National Cancer Institute, Bethesda), who presented data implying that at least some pseudogenes may not be pseudo at all, but coding sequences for secreted products.

When is a gene not a gene?

The basis for Jay's suggestion is a cDNA clone derived from the SWR/J mouse¹⁹ and whose sequence is broadly homologous to those of the *H-2K* and *H-2D* genes up to the transmembrane region. Here, through a series of substitutions and deletions, it encodes hydrophilic residues instead of hydrophobic ones, and moreover contains a stop codon. The cDNA was originally synthesized by reverse transcription from liver cell mRNA, and Jay now reports that liver is the only tissue in which the gene is transcribed; thus the tissue distribution of its product would be quite unlike that of the known *H-2K* and *H-2D* products, which are ubiquitous. This, and some recent (and rather controversial) evidence that liver transplants may suppress graft rejection²⁰, led him to propose a function for the product in the induction of immune tolerance. At this stage, however, there is only indirect evidence that the transcript is actually translated or secreted: Jay finds a

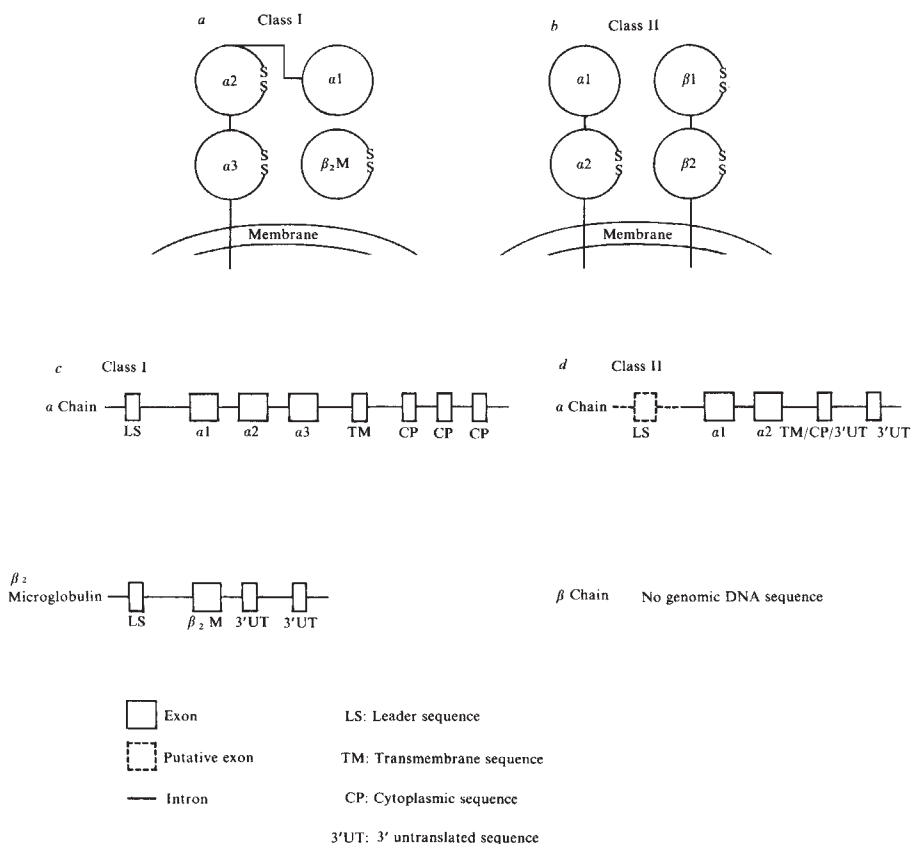


Fig. 2 Schematic diagrams of the class I (a) and class II (b) antigens, drawn so as to emphasize the probable homology of their quaternary structures. Each molecule has four domains: $\alpha 1$, $\alpha 2$, $\alpha 3$ and β_2 microglobulin for class I molecules, and $\alpha 1$, $\alpha 2$, $\beta 1$ and $\beta 2$ for class II molecules. a, Class I antigens are composed of an α -chain that in the case of the classical antigens is very highly polymorphic. The α -chain spans the plasma membrane, but can be expressed on the cell surface only in association with β_2 -microglobulin, a highly conserved molecule encoded outside the MHC and indeed on a different chromosome (2 in mouse, 15 in man). β_2 -microglobulin, which does not span the membrane, was early recognized as having considerable homology in general domain structure with immunoglobulin (it has been described as an "orphaned immunoglobulin domain"). More recently, the $\alpha 3$ domain of the β -chain has also been shown to be homologous to immunoglobulin³. b, The two chains of the class II antigens are both encoded in the MHC, and both span the plasma membrane. The α -chain is relatively non-polymorphic, the β -chain highly polymorphic. Homology to immunoglobulin has been demonstrated in the $\alpha 2$ domain (A. Korman, Harvard University; D. Larhammar, Uppsala University) and in the $\beta 2$ domain^{4,6}. The domain boundaries of the proteins correspond to the exon-intron boundaries of the DNA of all chains for which genomic clones are available (c and d).

protein of the right size in serum, but it has not been fully characterized and is not known to be secreted by liver cells. Although for the time being participants at the Oxford meeting were inclined to be sceptical about the proposed function of the liver transcript, it did lead to a reexamination of the criteria used to identify pseudogenes; and it focused attention on some interesting features of the 3' end of the MHC gene sequences.

A pseudogene can be identified by: (1) the deletion of coding sequences; (2) the presence of termination codons in coding sequences; and (3) non-conservation of the RNA splicing signals at intron-exon junctions. The pseudo status of the *HLA* gene sequenced by Biro, which in any case has a frameshift mutation in the first exon, is by these criteria unassailable. The mouse sequence of Steinmetz *et al.*¹¹ is a more ambiguous case. Its departures from or-

thodoxy do not begin until its transmembrane exon, which encodes one charged residue that ought to be hydrophobic, and contains a termination codon. The remaining mutations — another stop codon and a non-conserved splice site — occur 3' to the transmembrane exon; so it is not impossible that this pseudogene encodes a secreted product.

A question of paramount importance is thus how cloned genes can be assayed quickly for function. This question was raised by Hood in the context of an interesting drawback that has just come to light in the use of transfected cells for this purpose. The technique depends on the ability of transfected mouse L cells to express the cloned gene on their surfaces, where the product can be detected serologically^{12,13} or indeed function as a target for *H-2*-restricted virus-specific cytotoxicity²¹. However, Goodenow now

reports that transfection of L cells with cloned DNA containing only the 5' half of a class I gene has resulted in the surface expression of a new class I molecule. How the incomplete gene contrived to pick up the necessary 3' half containing the transmembrane sequences is a mystery. To avoid being misled by such rescued genes in the future, Hood suggests sequencing the most variable regions of cloned genes, synthesizing the corresponding peptides, raising monoclonal antibodies against them, and using the antibodies to test for expression on tissue cells. While this would not abolish false positives (because of the possibility of cross-reacting determinants), it should reduce them. (Not everyone, of course, has access to all the necessary facilities).

But the most exciting application of transfected L cells will be in the elucidation of MHC antigen functions by reverse genetics. Both Hood and Flavell gave notice of their intention to manipulate the sequences and reshuffle the exons of cloned genes in order to establish what these manoeuvres will do to the functions of their products.

Of particular interest is the part played by the cytoplasmic domains in immune function, because (for example) if the recognition of an MHC antigen triggers a signal from the recognized cell, it must be the cytoplasmic domains that mediate the release of the signal. Furthermore, there are hints of alternative splicing or termination sites at the 3' end of the MHC sequences. Malissen *et al.*¹⁵ have suggested, rather indirectly from comparisons of human genomic with mouse cDNA, that the pattern of splicing of the three cytoplasmic exons may vary — an idea that is now supported by comparisons with human cDNA (J. Trowsdale, ICRF); and Steinmetz *et al.*¹¹ have evidence that the last intron may not always be removed from mouse transcripts of class I genes. There is no easy way to check what is normally transcribed from most of the genomic clones, because the probes used to obtain them are in many cases from a different gene or even species. This also makes it difficult to check for cloning artefacts that might account for some of the unexpected features of the genes. To resolve these questions, it will now be necessary to go back and synthesize cDNA from the messengers transcribed from the cloned genes themselves in the cells that normally express them.

Class II genes and conservation

Cloning of the class II genes has only just begun, but it is clear even at this stage that unlike the class I genes they correspond more nearly in number to their known products. Probes for the α -chain genes have consistently revealed only one strongly hybridizing band in man (A. Korman, Harvard University; H. Ehrlich, Cetus; D. Larhammar, Uppsala University; J. Lee, Imperial Cancer Research Fund, London; C. Wake, Geneva

University) and in mouse (Lundt, NIMR, London; Steinmetz; Seidman). The strongly hybridizing band in man has been detected using a *DR α* gene, which is homologous to mouse *IE α* . Three additional bands have been detected by Trowsdale under relaxed hybridization conditions. One of these may be the *DCI* gene — the putative human homologue of the mouse *IA α* : it is present in a different genomic clone from that containing the *DR α* gene, and may be homologous to a cDNA clone identified by preliminary sequence analysis as *DCI* (C. Auffray, Harvard University). The identity of the third band is unknown. There is some uncertainty about how many human class II α genes to expect, so three would not be a major surprise. But two surprises have been delivered by human α -chain clones. Korman and Lee have shown that there are two polyadenylation signals in the 3' untranslated cDNA sequence, which may explain preliminary evidence from Lee for *DR α* mRNAs of different lengths. The significance of this finding is not known.

The second surprise is a *DR α* cDNA clone containing a termination codon before the hydrophobic sequence (Larhammar). This could be interpreted as corresponding to a transcribed pseudogene, or to a real gene encoding a secreted product; or it may be a cloning artefact. The last possibility has to be taken particularly seriously in this case, because of the small size of the *DR α* family.

The absence of large numbers of extra α loci may not be entirely surprising, in the light of the preceding evolutionary speculations, since the α chains of the class II antigens are relatively non-polymorphic. But the β -chains may have as many as

20–40 alleles per locus, yet so far neither man (L. Rask, Uppsala University; E. Long, Geneva University; and ref. 22) nor mouse (Lundt, National Institute for Medical Research, London; L. Clayton, Massachusetts Institute of Technology) seems to have more than two or three hybridizing sequences. If polymorphism depends on frequent crossing-over, or a reservoir of variant sequences for gene conversion, why is the class II gene family not as large as the class I family? There are two possible answers. The preliminary estimates on gene number may be wrong (evidence from protein sequences suggests six to eight genes, rather than two or three in man²³); or hybrids between different α - and β -chains may conceivably make a significant contribution to phenotypic polymorphism, obviating the need for so large a genetic source.

It is, however, worth noting that not everyone would expect the high polymorphism of the MHC to be associated with rapid evolution. J. Klein has argued from genetic analyses of wild mice that MHC polymorphisms are extremely stable in evolution; that neither mutation nor recombination is frequent; and indeed that the different allelic variants of the MHC antigens may have diverged before mouse diverged from man²⁴. In short, the mammalian MHC must have undergone a period of expansion and divergence early in its evolution, but long ago have become maximally adapted to deal with the prevailing pathogens. It may therefore be necessary to look elsewhere for an evolutionary explanation for the structure of the MHC.

One possibility that has to be faced is that it may have no adaptive significance at all, and merely represents a tableau of the processes everyone has assumed must underlie protein evolution. It is generally accepted that families of structurally related but functionally distinct genes must have evolved by duplication and divergence (as indeed so many of the genes of the immune system presumably evolved from a primate immunoglobulin-like domain); and there is every reason to suppose that during the period of divergence one of the duplicated genes would have become non-functional, through an accumulation of the termination codons, frameshifts and deteriorated splicing signals that characterize pseudogenes. Gene conversion offers a mechanism for correcting many such disabling mutations in one step — thus enabling the diverging gene to be 'tested' at intervals for new functions²⁵. The random introduction of new stop codons or poly(A) addition sites could produce shorter or longer molecules, or transform a membrane-bound to a secreted molecule if they occurred before a region encoding hydrophobic residues. Since we assume that such processes are random, we should not necessarily expect every transcribed or even translated gene to contribute to survival.

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REVIEW ARTICLE

Repetitive sequence transcripts in development

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Interspersed repetitive sequences are represented widely in animal cell nuclear RNAs, in the poly(A) RNA stored in eggs and in some mRNAs. Their expression is developmentally modulated. Although the genomic location of repetitive sequences may change rapidly during evolution, the patterns of their transcription suggest a variety of possible functions.

THE repetitive DNA sequences of the animal genome are extensively represented in cellular RNA. The patterns of repeat sequence expression have been known for some years to vary during development, and from tissue to tissue¹. However, until cloned probes became available, it was usually not possible to investigate specific repeat sequence families, or to distinguish quantitative developmental changes in repetitive sequence expression (that is, changes in the number of repeat transcripts of given sequence families) from qualitative changes (differences in the identity of the repeat sequence families represented in RNA). The availability of repetitive sequence clones, each representing a single family of more or less homologous sequences, has now opened new areas of investigation. The typical animal cell contains several very different kinds of repetitive sequence transcript, including (1) mRNAs derived from families of related structural genes, such as the histone or actin mRNAs; (2) the small nuclear RNAs (snRNAs), usually only a few hundred nucleotides or less in length and transcribed from members of certain specific repetitive sequence families; and (3) heterogeneous nuclear RNAs (nRNAs) and some cytoplasmic poly(A) RNAs that include repeat sequence transcripts. The latter RNAs are generally relatively large [1 to >10 kilobases (kb)], and are believed to be transcribed by polymerase II. This brief review is focused mainly on this heterogeneous class of repeat transcripts, the very existence of which remains a major puzzle of molecular biology.

Interspersed organization and diversity of transcribed repetitive sequences

The population of RNAs present in the cell nucleus has a predominantly interspersed sequence structure. Covalent linkage of various short repeat sequence elements, typically only a few hundred nucleotides long with transcripts of single copy sequences from the same genomic transcription unit has been shown for the nuclear RNA of HeLa cells²⁻⁵, sea urchin embryos⁶ and rat ascites cells⁷. An interspersed sequence organization is also characteristic of the maternal poly(A) RNA stored in sea urchin^{8,9} and amphibian¹⁰ eggs. The sea urchin egg poly(A) RNA transcripts that include repetitive sequences average 5–10 kb in length and consist of repeat elements a few hundred nucleotides long, interspersed with sequences hybridizing to single copy DNA (ref. 9 and J.W.P. *et al.*, unpublished data). Both complements of many or all of the transcribed repetitive sequences are represented in the interspersed RNAs, though generally in different molecules. Therefore the egg poly(A) RNA molecules can be renatured to form branched multimolecular structures that can be visualized in the electron microscope (Fig. 1a).

Although the repeat-containing poly(A) RNAs of sea urchin and *Xenopus* eggs include single copy or near-single copy

sequences later found on embryo polysomes^{9,10}, it is not yet clear whether any of the repeat elements themselves are included in polysomal embryo mRNAs (see, for example, refs 9, 11–15). Interspersed repeat transcripts have been demonstrated unequivocally in the mRNA of the cellular slime mould *Dictyostelium*¹⁶⁻¹⁸. Firtel and his associates identified two specific families of short repeat sequences at the 5' ends of sets of mRNAs, most of which consist of otherwise unrelated single copy transcripts^{16,17}. However, in at least one of these *Dictyostelium* mRNA families, repeat sequences are represented only in an asymmetric fashion, so that although the transcribed repeats are dispersed in the genome only one complement is found in the mRNA¹⁷. There is also evidence that in human lymphoblastoid cells, about half the polysomal poly(A) RNA contains repeat sequences, some of which may belong to the high-frequency *Alu* repeat family¹⁹. The *Alu* family²⁰ is also expressed prominently in HeLa cell heterogeneous nRNA²¹. Members of the *Alu* repeat sequence family apparently contain promoters for polymerase III and are represented in a major family of snRNAs²²⁻²⁵, the 7S snRNAs.

Almost nothing is known of the diversity of the transcribed repeats represented in the interspersed RNAs of human and other mammalian cells. Most recent studies have focused on only one of the large number of repetitive sequence families present in these genomes (J. W. Roberts, R. Hudspeth, E.H.D. and R. J. Britten, unpublished data and refs 26, 27), the *Alu* repeat family or (in other species) its close relatives. However, several different repeat sequence families are represented in the poly(A) RNA of human lymphocytes²⁸. Direct measurements^{8,9,29} show that in the sea urchin, at least a few hundred different repeat sequence families are represented prominently in egg poly(A) RNA. Of importance in considering the significance of repeat sequences is the recent finding that only a few members of each family may actually be transcribed to any extent (J.W.P. *et al.*, unpublished data). Figure 1b displays sea urchin egg and embryo RNA blots carried out with the separated strands of a cloned probe containing a typical short repetitive sequence. Each of the several transcript species visualized derives from a different member of the large repeat sequence family represented by the probe. Both strands of given repeat sequences occur in the interspersed RNAs, as indicated in parts a and b of Fig. 1, because some of the diverse transcription units including these repeats are oriented in one direction with respect to these particular repeat sequences, while others are oriented oppositely.

Developmental regulation

Complex as it is, the pattern of interspersed repeat sequence transcription is by no means constitutive and unchanging. Comparison of the second and third tracks in Fig. 1b reveals a qualitative developmental change in the expression of the

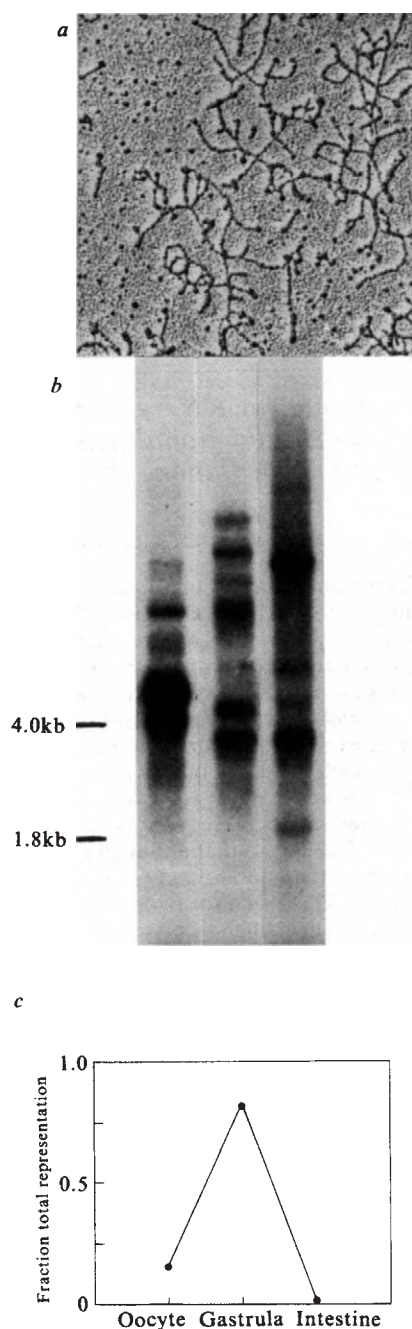


Fig. 1 Characteristics of egg and embryo poly(A) RNAs containing interspersed repetitive sequences. *a*, Poly(A) RNA of mature *Xenopus* oocytes, renatured to RNA C_{0t} 600 and spread for electron microscopy from 80% formamide¹⁰. A distance of 1 cm represents a single-stranded RNA length of 870 nucleotides. The branched multimolecular RNA networks are held together by base-paired repetitive sequence duplexes, and include about 70% of the total oocyte poly(A) RNA. Fedoroff *et al.*⁴ showed earlier that structures similar to these are formed when nuclear RNA is renatured. *b*, RNA gel blots using separated strands of a short interspersed repetitive sequence clone called CS2109B. Left, 'L' strand reacted with egg poly(A) RNA; centre, the complementary 'U' strand reacted with the same RNA; right, the same strand as used in the centre track reacted with blastula stage embryo poly(A) RNA; the exposure of the blastula track is several-fold lower (unpublished experiments of J.W.P., R. J. Britten and E.H.D.). *c*, Relative extent of expression ('per cent representation'³⁰) of the 'U' strand of the 2109B repeat sequence in gastrula nuclear RNA and adult intestine cell nuclear RNA, compared with egg RNA, from molecular titrations carried out with the cloned probe³⁰.

repeat family, which implies the transcription in blastula stage embryos of additional members of the family. Scheller *et al.*³⁰ found for each of nine sea urchin repeat clones a unique pattern of representation in embryo nuclear RNA, which differed in adult intestine cell nuclear RNA and again in egg RNA. Representative data are shown in Fig. 1c for the same clone as used for the RNA gel blots in Fig. 1b. In fact, the embryo and adult intestine nuclear RNAs of the sea urchin are much more easily distinguished by their differing contents of specific repeat sequence transcripts³⁰ than by their largely overlapping sets of single copy sequence³¹⁻³³.

A second system in which developmental regulation of repeat sequence transcripts has been observed is *Dictyostelium*. One of the mRNA sets sharing a common repeat sequence¹⁷ is very rare in vegetative cells but becomes increasingly prevalent as development proceeds, beginning at ~5 h (A. R. Kimmel and R. A. Firtel, personal communication). RNA gel blots carried out with single copy probes obtained from two of these transcripts show that both appear at about the same time. The developmental change in the abundance of this set of mRNAs is about 10-fold. Zuker and Lodish¹⁸ also reported developmentally regulated sets of interspersed cytoplasmic *Dictyostelium* poly(A) RNAs, each of which is defined by the presence of a particular repeat sequence. At least some members of these sets are 'coordinately regulated', to the extent that in one case, the multiple transcripts cannot be detected in vegetative cells but appear by 5.5 h of development, while another set appears only by 15 h.

Developmental regulation of a different kind of repeat sequence transcript has been reported in *Drosophila*. These transcripts are exemplified by the poly(A) RNAs carrying the *copia* sequence. The *copia* repeat sequences are about 5 kb long, and are apparently able to transpose at a relatively rapid rate, as their location in the genome as well as their number differ among *Drosophila* strains and among tissue culture cell lines^{34,35}. Structural features of the *copia* element and its sites of insertion are reminiscent of other eukaryotic and prokaryotic transposable elements³⁶⁻³⁸. The *copia* sequence is transcribed into cytoplasmic poly(A) RNAs ~2 and 5 kb long, and at least the 2 kb RNA can be translated, and thus, is thought to be an mRNA³⁸. The *copia* transcripts differ basically from the repeat-containing RNAs thus far mentioned in that they do not have an interspersed sequence structure. The 2 and 5 kb transcripts begin and end within the *copia* repeat sequence element³⁵. Flavell *et al.*³⁸ and Scherer *et al.*³⁹ have reported that the level of *copia* transcripts is modulated during development. These transcripts are not detectable in early embryos but appear after 10 h, and they are most concentrated in larvae. Developmental patterns of regulation have also been reported for transcripts of at least three other *Drosophila* long repeat sequence families that appear to be transposable and to share the general features of the *copia* sequences^{39,40}.

Are repeat sequence transcripts biologically significant?

The observation that repeat sequence expression is correlated with developmental change does not, of course, demonstrate function, though repeat sequences might seem much less interesting were their transcription non-existent or invariant. Clearly there are diverse kinds of developmentally regulated repeat sequence transcript, the properties and potential significance of which must differ in basic ways. The 5' location of the repeats and their asymmetric representation in the *Dictyostelium* mRNA sets studied by Kimmel and Firtel¹⁷, and the apparent coordinate expression observed both for these transcripts and the mRNA sets studied by Zuker and Lodish¹⁸, led both groups to suggest that *Dictyostelium* mRNA repeat elements serve as coordinate regulatory sequences⁴¹. Such arguments are unlikely to pertain to *copia*-like repeats because these elements mainly promote transcription of their own sequence. On an evolutionary scale, of course, the presence of transposable elements that

include promoters could be of fundamental importance, because readthrough transcription of adjacent genes might occur as the result of an insertion event. A very instructive example is found in the yeast ROAM mutants studied by Errede *et al.*⁴², who concluded that insertion of a Ty-1 transposable repeat element (and perhaps additional flanking sequences) in the 5' region of the iso-2-cytochrome *c* gene causes this gene to be brought under the positive control of diffusible regulators from the mating-type locus.

There are two opposing attitudes towards the significance of the interspersed short repeats lying in and around the transcription units of animal genomes⁴³⁻⁴⁵. As proposed originally by Britten and Davidson⁴⁵, interspersed repeats seem to 'diffuse' around the genome during evolution. Recent relevant evidence includes the findings that the family sizes of specific interspersed repetitive sequences differ strikingly between related sea urchin species⁴⁶; the mammalian *Alu* repeat sequences and their homologues are flanked by brief terminal repeats similar to those that surround known transposons²³; and in human⁴⁷ and monkey⁴⁸ cells, these same sequences can be recovered from closed circular extrachromosomal DNA, just as *copia* sequences can be found in closed circular DNA of *Drosophila* cells⁴⁹. Thus one way of interpreting the developmental modulations observed in interspersed repeat transcripts is simply to conclude that among the hundreds of thousands, or, in some genomes, millions of short repeat elements are some that happen to have been transposed, harmlessly, into developmentally modulated transcription units. Though functionless, these repeat elements would be transcribed merely because they lie in the path of the polymerase. Thus, for example, interspersed repeats are known to be present in intervening sequences of several genes, including those for vitellogenin⁵⁰, growth hormone⁵¹ and conalbumin⁵². Such repeats would be included in the primary mRNA transcripts from these genes, and their expression would clearly appear developmentally modulated, because those genes are active only in certain cell types. The alternative view is that it may be premature to conclude that because molecular biology has not yet demonstrated a functional role for interspersed repeat transcripts, none exists in the cell. Hidden within the vast networks of sequence homology formed by dispersed genomic repeat families there might be small subsets of homologous sequence elements that serve as receptors for

diffusible regulators and are involved in coordination of gene activity as proposed earlier⁴¹. The proposition that members of interspersed repeat families are undergoing a continuous, evolutionarily rapid redistribution suggests that many insertions and deletions are without phenotypic effect, and thus it is reasonable to suppose that even if some repeat elements of large families were to perform regulatory functions of some kind, most of their homologues are without function.

The interspersed transcripts stored in mature eggs are especially interesting from the standpoint of possible function, because they represent the major part of the mass of the egg poly(A) RNA, and result from accumulation during a long period of synthesis in oogenesis. They are evidently destined for use during embryonic development. These transcripts in some ways resemble mRNAs, although they are stored in the oocyte cytoplasm¹⁰. The repeat elements themselves are not likely to be translatable (ref. 29 and J.W.P. *et al.*, unpublished data) and could be located in long 3' mRNA 'tails', or in internal positions such as intervening sequences that have not been processed out. Perhaps these interspersed maternal transcripts can be used in embryogenesis only after further modification, in contrast to the maternal mRNAs capable of immediate translation that are clearly also present in the egg¹. Embryonic processing of maternal transcripts could be an important developmental regulatory event, or these transcripts could play other developmental roles altogether, as suggested by Costantini *et al.*⁹. Other recent speculations have centred on the intermolecular complementarity of the repeat elements in mRNA, and of some snRNAs with mRNAs. Intermolecular complementarity could provide the molecular basis for splicing or other processing decisions (for example refs 53, 54).

In summary, a variety of interpretations have so far resisted exclusion. Despite their ubiquity, their quantitative prominence, their apparent developmental regulation and the amount of interest they have aroused, the repetitive sequence transcripts of animal cells remain a phenomenon in search of a physiological meaning.

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Note added in proof: Calvet *et al.*⁵⁵ have demonstrated by *in situ* psoralen cross-linking that at least U1 and U2 snRNAs are base paired to mRNAs within the cell nucleus.

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ARTICLES

Climatic significance of the hydrogen isotope ratios in tree cellulose

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A distinct relationship exists between the δD values of cellulose carbon bound hydrogen in trees and average annual temperature for a variety of trees from a wide range over North America. The slope of the $\delta D/T$ relationship is $5.8\% \text{ } ^\circ\text{C}^{-1}$. Samples of annual precipitation covering much the same geographical range as the trees exhibit a comparable temperature coefficient of $5.6\% \text{ } ^\circ\text{C}^{-1}$. Such growth-site conditions as poor drainage and/or low relative humidity seem to perturb the spatial $\delta D/T$ relationship. However, our data indicate that suitable tree growth sites are more the rule than the exception.

A GROWING body of evidence suggests that variations in the D/H ratios of the carbon-bound hydrogen of tree cellulose primarily reflect variations in the D/H ratio of associated meteoric waters as manifested in the isotopic composition of the leaf water¹⁻³. Because there is a generally recognized relationship between climatic temperature and the stable hydrogen and oxygen isotope composition of meteoric water⁴⁻⁶, there is an expectation that the δD variations of tree cellulose C-H hydrogen will reflect variations in climate

$$\delta D = \left(\frac{R_{\text{sample}}}{R_{\text{standard}}} - 1 \right) \times 1,000, \text{ where } R = D/H \text{ ratio.}$$

The standard is SMOW (standard mean ocean water as defined in ref. 7). Such a climatic 'signal' has already been identified for tree cellulose $^{18}\text{O}/^{16}\text{O}$ ratios⁸⁻¹⁰.

The few studies that have been done on the climatic significance of tree cellulose C-H hydrogen δD variations¹¹⁻¹⁴ have not been completely satisfactory, largely because of experimental methodology^{1,15,16}. With the development of techniques that yield highly reproducible δD values (refs 3, 17), the opportunity has arisen for further investigations into the climatic significance of the D/H ratios of tree cellulose carbon-bound hydrogen (note that our analytical method has a δD precision of $\pm 2\%$).

When considering the climatic significance of δD variations in trees, it is important to keep in mind that there are two scales of climatic change which are of interest—spatial and temporal. The δD variations in trees offer the possibility that both spatial and temporal climatic signals can be discerned, because trees are geographically widespread and also contain an internal chronology which may isotopically record local climatic change. This article is concerned with the climatic significance of tree δD values on the spatial scale. The question of the climatic significance of δD variations within the chronologies of individual trees will be considered elsewhere.

Results and discussion

Twenty-five trees from 22 sites throughout North America were obtained and analysed for their cellulose C-H hydrogen δD value by the renitration method³. The outermost four or five rings made up the samples which were analysed. Because of the different times at which the various trees were felled, the total interval spanned by the samples is AD 1961-75. Sample descriptions can be found in ref. 3. The locations of the trees as well as the North American sites of the International Atomic Energy Agency (IAEA) precipitation stations are plotted in Fig. 1. It can be seen in Fig. 1 that the tree samples cover much the same range of geography and climate as the IAEA stations. Thus, if the δD variations of the tree cellulose C-H hydrogen

reflect the δD values of local precipitation, the two should have similar relationships to spatial variations in climatic temperature.

The average annual temperature and precipitation δD values of the IAEA sites of Fig. 1 are plotted in Fig. 2. The relationship between these two variables is readily apparent, and a linear regression of these data yields the expression:

$$\delta D = 5.6T(^{\circ}\text{C}) - 120 \quad (1)$$

with a correlation coefficient of 0.90. The value of 5.6 for the slope of this equation is the same as that found by Dansgaard⁴ for maritime and Arctic stations, although the intercept of the North American data of Fig. 2 is more negative by about 20%.

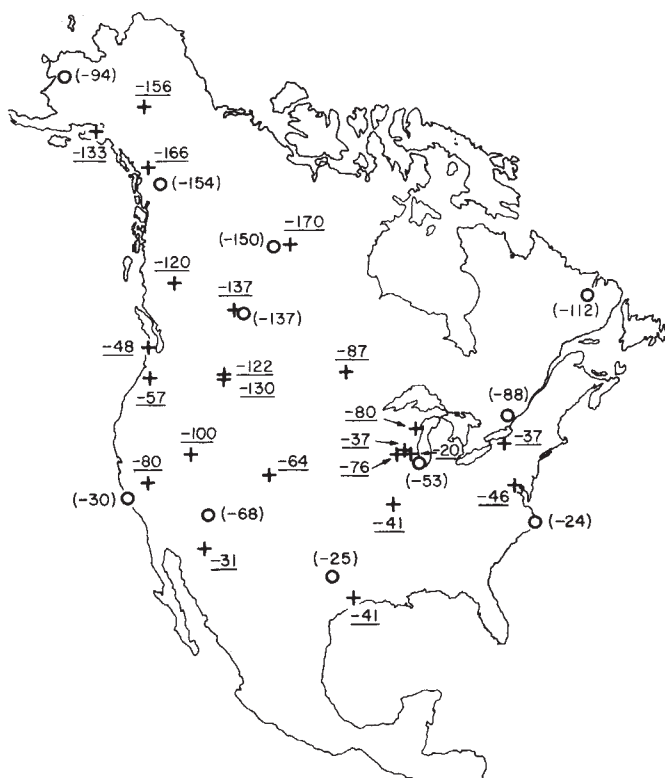


Fig. 1 The geographical distribution of the δD values of tree samples analysed for this work. In addition, the δD values of annual precipitation at the 11 North America IAEA stations are depicted. +, Tree sites; O, IAEA stations. δD values of the trees are underlined, while the δD values of the precipitation at the IAEA stations are shown in parentheses. Note that there is a general decrease in δD values of both the trees and the precipitation from coastal to inland sites and from south to north.

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The δD values measured for the trees and the average annual temperatures of nearby meteorological stations are listed in Table 1 and plotted in Fig. 3 which shows that there is a relationship between the δD values and annual temperature although some scatter is also evident. The five circled points in Fig. 3 seem to lie significantly above the general 'trend line' of the data and may be unusual from the standpoint of the conditions in their environments of growth. For example, UBLM-2 is a willow tree which grew in a marsh in Madison, Wisconsin. The waters in the soil in which this tree grew were analysed and found to have a δD value of -32% . This value is considerably more positive than the average annual precipitation in the area, the latter of which has a δD value of about -60% . Consequently, either the meteoric water incorporated by the tree had undergone significant evaporation as it slowly infiltrated into the poorly drained soil, or the water represented predominantly summer precipitation which is commonly more positive than the average annual precipitation¹⁸. In either case, the δD value recorded by the tree would not be representative of average annual precipitation and therefore would probably not reflect average annual temperature.

Samples MNY-GA-2, MNY-RM-5 and MNY-BO-7 (encircled in Fig. 3) grew in a marsh in New York. The waters utilized by these plants were not available for analysis, but conditions similar to those noted for the UBLM-2 site were possibly present at the site of these three trees. They may then have incorporated waters with δD values which were more positive than the average annual δD values of precipitation in the area and therefore the trees would not have δD values representative of the local average annual temperature.

The fifth point encircled in Fig. 3 is AW-BO-1 a bur oak which grew in a pasture in southeastern Wisconsin amidst a small stand of oaks. The analysed δD value of water from a nearby stream was -62% . This water value is similar to δD values measured for water from the Wisconsin River and from a river near Oconto, Wisconsin. In the latter two cases, trees (WR-2, OM-2) which grew in soils immediately adjacent to these waters had δD values in the range -76 to -80% . Consequently, if AW-BO-1 had incorporated waters with a δD value of -62% , it would have been expected to have a δD value around -75% . In fact AW-BO-1 has a δD value of -23% . Unless there is some unknown biochemical or physiological factor present in bur oaks which is causing this compara-

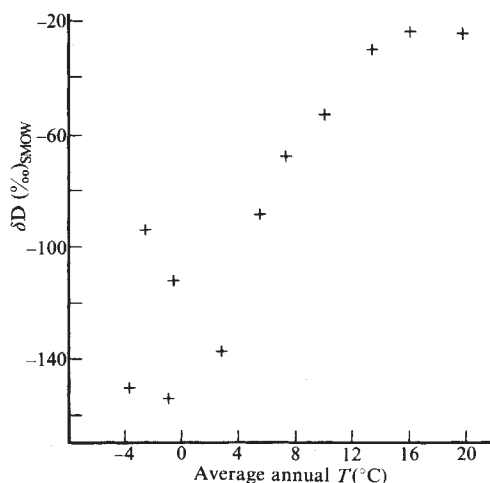


Fig. 2 A plot of the δD values of annual precipitation against annual temperature for 11 available North American IAEA stations. The linear regression of these data yields the equation: $\delta D = 5.6T (^{\circ}\text{C}) - 120$. The correlation coefficient is 0.90.

tively positive δD value, it appears that this tree incorporated waters with δD values more positive than the average annual value. As with UBLM-2, this might be the case if the root system were sufficiently near the surface to cause the tree to use only summer precipitation. Alternatively, the infiltration rate of water into the soil may be slow enough in the environs of the tree to allow significant increase in δD values of precipitation due to evaporation while on the soil surface (see ref. 19).

As discussed in ref. 19, this soil surface evaporation could cause a greater increase in δD values of the water than would occur from transpiration alone. Thus, significant above-ground evaporation of precipitation might cause tree δD values to be more positive than otherwise expected. The relationships of these more positive tree δD values to annual temperature in a spatial $\delta D/T$ plot would probably differ from that of other sites at which no above-ground evaporation of precipitation had occurred.

In short, the circled points in Fig. 3 are interpreted to be representative of sites at which trees incorporated soil water that was more positive than the δD value of average annual

Table 1 δD of cellulose nitrate and approximate average annual temperature in region of growth site—all sites are North American

Sample no.	Name	Location	Average annual $T (^{\circ}\text{C})$	Cellulose nitrate δD
OPW-DF-1	Sitka spruce	Olympic Peninsula, Washington	11.3	-48
Col-DF-1	Douglas fir	Jefferson Co., Colorado	10.5	-64
MNY-GA-2	Green Ash	Montezuma National Wildlife	8.2	-33
MNY-BO-7	Bur Oak	Refuge,	8.2	-34
MNY-RM-5	Red Maple	New York	8.2	-45
RE-CO-2	Chestnut oak	Reston, Virginia	14.0	-46
BCT-12	Pine	Seeley Lake, British Columbia	3.4	-120
MO-O-2	Oak	Owensville, Missouri	12.6	-41
AW-BO-1	Bur Oak	Albion, Wisconsin	7.0	-20
FCA-WS-1	White Spruce	Fox Creek, Alberta	0.9	-137
SAS-JP-1	Jack Pine	Porter Lake, Northwest Territories	-3.5	-170
FA-As-1	Aspen	Fairbanks, Alaska	-3.3	-156
KSA-As-1a	Aspen	Anchorage, Alaska	2.1	-133
NT-1	Aspen	Brown Creek, Ruby Mts, Nevada	7.4	-100
MT-1	Birch	Flathead Lake, Montana	5.7	-130
MT-2	Maple	Flathead Lake, Montana	5.7	-122
UAZ-PP-1	Ponderosa pine	Catalina Mts, Arizona	19.3	-31
PO-DF-1	Douglas fir	Portland, Oregon	12.6	-57
H-2	Oak	Houston, Texas	20.2	-41
WM-B-1	Birch	Winnipeg, Manitoba	2.5	-87
WR-2	Maple	Spring Green, Wisconsin	7.0	-76
OM-2	White Birch	Oconto, Wisconsin	7.0	-80
UBLM-2	Willow	Madison, Wisconsin	7.0	-37
Yuk-W-1	White Spruce	Kluane Lake, Yukon Territory	-0.7	-166
BrPn	Bristlecone pine	White Mts, California	0.7	-80

precipitation. Thus, these samples suggest that site conditions may be very important in choosing trees for possible interpretation of geographical climatic variation. The scatter in Fig. 3 not accounted for by the circled points will be discussed below.

If the circled points in Fig. 3 are eliminated from consideration in the spatial $\delta D/T$ plot, the balance of the data produces an array of points for which a linear regression yields the expression:

$$\delta D = 5.8T (^{\circ}\text{C}) - 134 \quad (2)$$

The linear correlation coefficient is 0.88. The slope of this curve is very similar in magnitude to the slope of 5.6 obtained for the regression of North American IAEA precipitation data (Fig. 2). Because the tree sites and IAEA stations extend over much the same range of geography and climate (Fig. 1), it might be expected that a regression of the two sets of δD data against temperature would produce a similar slope, providing that the δD values of the tree samples used in the comparison reflect the δD values of annual precipitation at these sites.

Seven of the tree samples, OPW-DF-1, Col-DF-1a, RE-CO-2, BCT-12, MO-O-2, FCA-WS-1, and SAS-JP-1, included in the regression of the data of Fig. 3 have chronologies that extend back to at least AD 1931. They range in location from as far south as St Louis, Missouri, and Reston, Virginia, to as far north as Porter Lake, Northwest Territories.

In addition, these seven trees are located near meteorological stations whose instrumental temperature records also date from at least 1931. This suggests the possibility of plotting spatial tree δD variations against annual temperature for 5-yr intervals from 1931 to 1970. Such plots could determine whether or not there have been any important changes over this interval in the character of the $\delta D/T$ relationship as defined by the seven trees (see Fig. 4).

Because far fewer sites are included in Fig. 4 than in Fig. 3, it is statistically possible that the linear regressions of these data will not produce the same slope as that obtained from the regression of the data of Fig. 3. Thus, it does not seem to be possible to attach physical significance to any differences in slope between the large data group (Fig. 3) and its subset (Fig. 4). However, changes of slope between different 5-yr intervals in Fig. 4 may have a physical interpretation.

The equations for the linear regressions of the different 5-yr intervals are found in Fig. 4. The analytical error of $\pm 2\%$ for the measurement of δD values imposes an error on the regression slopes of Fig. 4 of about $\pm 0.3\% ^{\circ}\text{C}^{-1}$. Thus, with the exception of 1941–45 all the 5-yr intervals exhibit within experi-

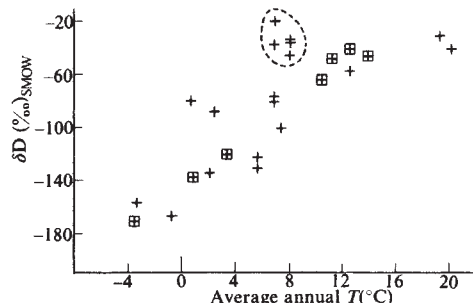


Fig. 3 A plot of the δD values of trees from a wide range over North America against the average annual temperature. The tree samples represent various intervals within the span 1961–75. The five points enclosed in the dashed circle represent trees which may have grown in conditions that could obscure a climatic interpretation of their δD values. The remaining 20 points define a trend with the following linear regression equation: $\delta D = 5.8T (^{\circ}\text{C}) - 134$. The correlation coefficient is 0.88. $\boxplus$, Trees in Fig. 4.

mental error the same spatial δD temperature coefficients. The large 1941–45 temperature coefficient of $8.6\% ^{\circ}\text{C}^{-1}$ seems to be a consequence of something other than normal analytical error. However, at present, we cannot explain it.

Notwithstanding the possibility that the spatial 1941–45 $\delta D/T$ slope is truly different from the others, the fundamental observation to be drawn from Fig. 4 is that over this entire 40-yr period, an impressively consistent pattern of declining tree δD values with declining spatial temperature has been maintained. This has occurred in spite of the differences in tree species and different relative ages of the trees. Thus, it enhances the conclusion that the δD values of trees can indeed be indicators of spatial climatic differences.

Some of the possible causes of the scatter in the 20 'well-behaved' data points of Fig. 3 should now be considered. It is possible, for example, that trees growing in regions of low humidity may exhibit comparatively positive δD values due to a high degree of deuterium enrichment in the leaf water during transpiration. This would tend to shift the cellulose carbon-bound hydrogen δD value above the general trend of the data of Fig. 3, even if the tree is using soil water whose isotopic composition is equal to that of the average annual precipitation³. The relatively positive position of the BrPn point in Fig. 3 may be explained by this effect, as the average growing season

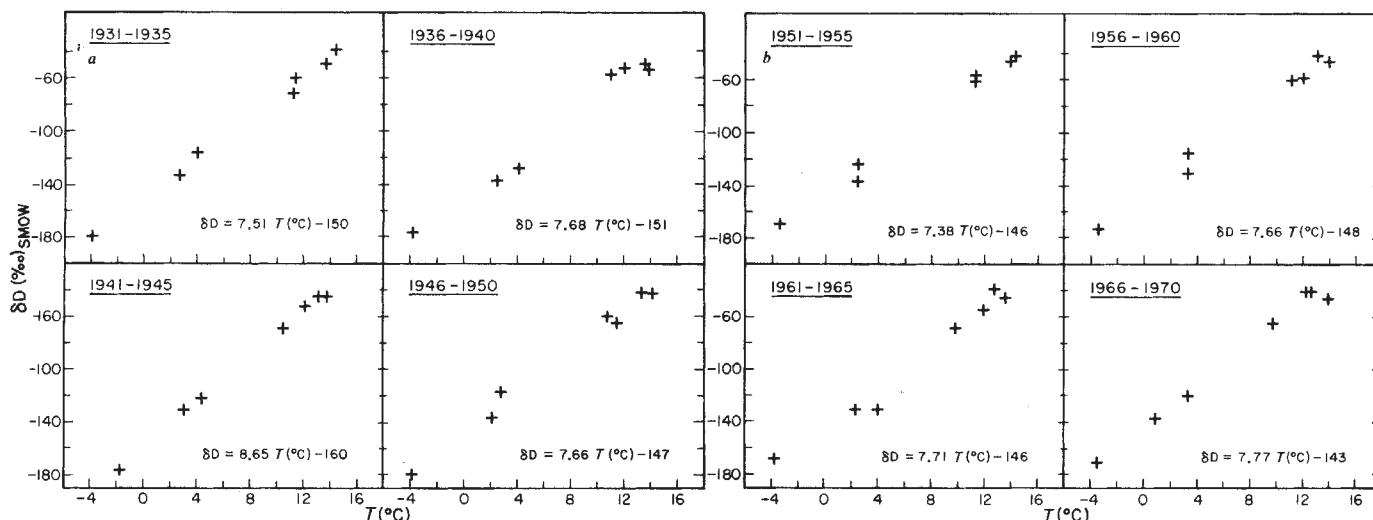


Fig. 4 a, Comparisons of the δD values of seven trees from a wide range over North America (OPW-DF-1, Col-DF-1a, RE-CO-2, BCT-12, MO-O-2, FCA-WS-1, and SAS-JP-1) with average annual temperature. The comparisons are made for different corresponding 5-yr intervals. The equations of the linear regressions of the data are shown within the plots of each of the 5-yr intervals. The pattern of declining δD values with declining temperature is remarkably consistent for the trees and the intervals depicted. b, Same trees as in a except that four different 5-yr intervals are compared. Again, the overall pattern of declining δD with declining temperature is maintained throughout these intervals in spite of the differences in species and relative ages of the trees.

relative humidity in the region of BrPn is only about 45% (ref. 3). Interestingly, it may eventually be possible to correct for the deuterium enrichment due to transpiration by analysing both hydrogen and oxygen isotope ratios in cellulose (see ref. 10).

A second source of scatter may arise from the fact that the surface temperatures used for comparison with the tree δD values are unweighted average annual temperatures. If the trees are incorporating annual precipitation, the δD value of this precipitation will probably be a weighted average of varying proportions of the various seasonal precipitations. Consequently, the unweighted temperature and weighted δD values may not always be uniformly related. Furthermore, the compared temperatures are those of meteorological stations which are not located at the exact site of tree growth. Thus, the pattern of geographical temperature variation of these stations may not be exactly analogous to that of the growth sites.

Biochemical or physiological differences between these 20 different 'well-behaved' trees could produce some, as yet, unrecognized differences in the net hydrogen isotope fractionation between leaf water and cellulose C-H hydrogen. Such differences might contribute to the scatter in Fig. 3.

There may also be trees among the 20 'well-behaved' samples that have not incorporated average annual precipitation. Instead, some trees may have utilized relatively more water from a single season. When plotted against annual temperature, the δD values of these trees might produce some scatter such as appears in Fig. 3.

Finally, there is likely to be scatter in the primary precipitation δD /temperature relationship (see Fig. 2). This scatter would be manifested in Fig. 3, if the δD values of the trees reflected the δD values of the local meteoric waters.

In spite of these possible sources of scatter in the relationship between tree δD values and annual temperature, Fig. 3 shows that such a relationship does exist. Furthermore, for the 20 'well-behaved' samples of Fig. 3, the relationship is quite close to that which might be expected if the δD variations of the trees mimic those of the local annual precipitation.

Thus, trees from the proper sites seem to record δD values that correspond to geographical climatic differences. Note that

the samples analysed for this work were collected by individuals who had little specific instruction regarding the relative merits of local tree sites. Thus, local site selection was largely random. From information supplied by individuals who collected the samples (see ref. 3), it seems that the 'well-behaved' sites are likely to be those in which there is an absence of stagnant soil, ground or surface water¹⁹.

Conclusion

A comparison of the geographical variation of the δD values of trees with the associated average annual temperature reveals an overall correlation for many sites and species of trees. The spatial linear temperature coefficient obtained for δD values from 20 widely separated tree samples in North America is $5.8\% \text{ } ^\circ\text{C}^{-1}$. This compares with a temperature coefficient of $5.6\% \text{ } ^\circ\text{C}^{-1}$ obtained from annual precipitation δD values at 11 North American IAEA sites. The similarity of these two coefficients is consistent with the idea that the δD variations of the trees reflect the δD variations of the local precipitation. The latter conclusion is supported by the spatial distribution of the tree δD values (Fig. 1). These δD values decrease from the coast to the interior and from south to north. This pattern of variation is analogous to that observed for meteoric waters over North America (Fig. 1).

Site conditions seem to be important in determining the δD values recorded by trees, and some site conditions can produce scatter in plots of annual temperatures against tree δD values on a spatial scale. However, the trees sampled for this work indicate that favourable growth sites are common.

The geographical temperature-tree δD correlation for seven widely separated North American trees shows a remarkable consistency over the interval 1931–70. This consistency is maintained in spite of the differences in both species and relative ages of the seven tree samples involved.

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β Decay and the origins of biological chirality: experimental results

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A spin-polarized low-energy positron beam has been used to set limits on asymmetric positronium formation in optically active molecules. No asymmetry was found at the 7×10^{-4} level in cystine and tryptophan, but a possible effect of $(31 \pm 7) \times 10^{-4}$ was found in leucine. A quantitative connection is made with the origin of biological optical activity.

THE amino acids and sugars on which terrestrial life is based show maximal optical activity, that is, with rare exceptions, they are composed of D-sugars in RNA and DNA and L-amino acids in proteins. This observation poses the questions: (1) Why should life be based on optically active substances? (2) Are there any causal mechanisms that would lead us to expect the selection of the L-amino acids in terrestrial organisms and is

this choice expected to be the one statistically preferred when all possible biospheres are considered? Question (1) has been discussed extensively^{1,2} and there is widespread agreement that life should naturally select an ordered system based on optically pure substances. However, question (2), still unanswered, is one of the important problems in chemical and biological evolution.

In the absence of any causal mechanisms, the presently observed complete optical purity would be the result of a random fluctuation in the presumed virtually racemic isomeric balance of the primordial Earth, followed by chemical and biological amplification. The probability of selecting a particular isomer would then be 50%. On the other hand, causal mechanisms can systematically produce an isomeric excess which, if large enough to compete with random fluctuations, may strongly bias the odds on which isomer will eventually dominate. To generalize our treatment of question (2) to all types of biospheres it is necessary to distinguish two types of causal mechanisms: local and universal. A local causal mechanism relies on some local asymmetry to produce a bias in the odds on which isomer dominates in that particular biosystem. By contrast, a universal causal mechanism produces a systematic bias in the odds in favour of one isomer dominating throughout all biosystems in which it is effective. All such mechanisms must be related to the weak interaction as this is the only interaction that universally violates parity conservation. No reproducible quantitative results are yet available for either the random or causal mechanisms. The description, theoretical analysis, and preliminary experimental results of a new method to investigate the most plausible universal causal mechanism, preferential radiolysis by electrons emitted in the β decay of radionuclides, is the subject of this and the accompanying article³. For completeness, we also present a brief discussion of the leading local causal mechanism, preferential photolysis or catalysis by circularly polarized sunlight.

Analysis of two causal mechanisms

Preferential photolysis by circularly polarized light: Asymmetric effects have been observed in the interaction between amino acid isomers and circularly polarized light⁴. In addition, a circular polarization (CP) of sunlight of 0.1% has been observed at dawn and dusk in an IR band (780–800 nm)⁵. However, a corresponding CP in the UV, needed for asymmetric photolysis, was not found, and thus it must be <0.01%. In addition, because CP is a result of parity conserving electromagnetic interactions in the atmosphere, it can only be a local causal mechanism, that is, it must be identically zero when averaged over one Earth rotation and over the Earth's surface, unless non-uniformities exist in time (morning compared with

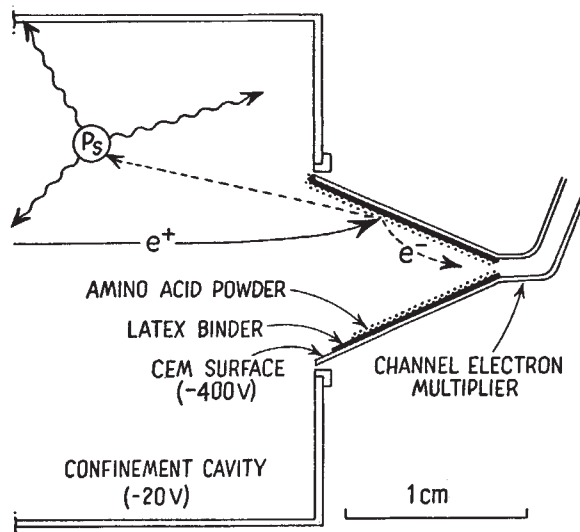


Fig. 2 Detail of the slow positron target region. A 0.5-mm thick layer of powder of a given sample was pressed on a binding layer of latex on the CEM cone. Measurements of Ps formation on the bare cone, the latex binder, and the amino acids confirmed that Ps formation occurred only in the amino acid layer. Secondary electrons are collected into the channel of the CEM. The triplet Ps formed in the amino acid powder lives a sufficient time to escape from the powder into the confinement cavity and annihilate into three γ rays with approximately the vacuum lifetime of 140 ns.

evening) and with respect to the Northern and Southern Hemispheres.

Preferential radiolysis related to electron helicity in β decay:

The radiolysing β decay electrons, on emission from the nucleus, possess a handedness or helicity (h), that is—there is a correlation between the direction of the spin angular momentum and the linear momentum. This correlation is expressed by the relation $h = \langle h_i \rangle = \langle \hat{s}_i \cdot \hat{p}_i \rangle$ where, for the i th particle, $\hat{s}_i$ is the Pauli spin matrix, $\hat{p}_i$ the unit momentum vector ($\hat{p}_i = \hat{p}_i/|\hat{p}_i|$), h_i is the helicity operator, and the angular brackets represent an average over all particles in the ensemble. Further, for the present discussion $\langle \hat{s}_i \cdot \hat{p}_i \rangle = \langle \hat{s}_i \rangle \cdot \langle \hat{p}_i \rangle$, that is the positron spin and momenta are decoupled. If, for example, all spins and all momenta in a beam are parallel ($|\langle \hat{s}_i \rangle| = 1$ and $|\langle \hat{p}_i \rangle| = 1$). Preferential radiolysis by β decay electrons represents what we have defined as a universal causal mechanism and the problem is to determine to what degree this handed radiation could asymmetrically radiolyse racemic mixtures of amino acids on the primordial Earth.

Many experiments have searched for helicity induced preferential radiolysis^{6–8} but no reproducible effect has been demonstrated. We feel that this is because none of the experiments approached the level of sensitivity necessary to observe the small asymmetric effects which are now predicted to occur³. These effects arise from the distortion in the electronic wavefunctions in optically active molecules. The distortion induced by coupling between spin and orbital motion in the bound electrons produces a helicity per unit volume or helicity density—denoted $h(\vec{r})$. From considerations of parity conservation (mirror symmetry) $h(\vec{r})$ is zero for symmetric spin-unpolarized molecules but $h(\vec{r})$ can be non-zero for spin-unpolarized dissymmetric molecules.

The spin-dependent exchange interaction between the molecular electrons and the incident β decay electrons, when the coupling between $h(e^-)$, the helicity of the incident electrons, and $h(\vec{r})$ is included, produces an asymmetry, A_R , in the respective rates of radiolysis of L and D isomers $R(L)$, $R(D)$. On either reversal of $h(e^-)$ ($h(e^-) \rightarrow -h(e^-)$) or interchange of the L and D isomers this asymmetry may be written as:

$$A_R = \frac{R^+(L) - R^-(L)}{R^+(L) + R^-(L)} = \frac{R^+(L) - R^+(D)}{R^+(L) + R^+(D)} = |h(e^-)| H_R(E, Z) \quad (1)$$

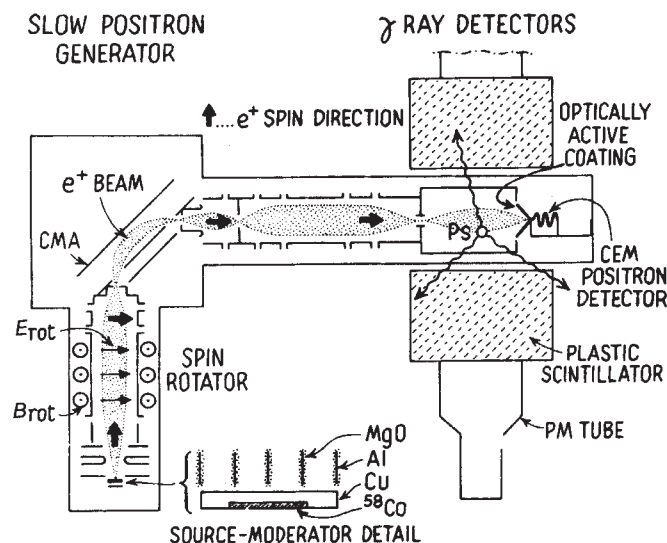


Fig. 1 The experimental apparatus used to measure asymmetries in triplet positronium formation. The beam consists of 3×10^3 positrons s^{-1} and initial helicity $h_0(e^+) = 0.21 \pm 0.02$ (ref. 13). The Wien filter spin rotator (crossed electric and magnetic fields) allows rotation of the average spin direction ($\langle \hat{s}_i \rangle$) of the beam with minimal effect on the average direction of the beam's momentum ($\langle \hat{p}_i \rangle$). Thus $h_0(e^+)$ may be continuously varied from +0.21 to -0.21.

Here (+, -) refers to $h(e^-) > 0$ or $h(e^-) < 0$ and from parity conservation $R^+(\text{D}) = R^-(\text{L})$ so that, for example, A_R may be written as:

$$A_R = [R^-(\text{D}) - R^+(\text{D})] / [R^-(\text{D}) + R^+(\text{D})]$$

H_R , an effect of the molecular helicity density $h(\vec{r})$, is essentially an average of $h(\vec{r})$ weighted by the Coulomb interaction between the projectile (β) electron and the target (molecular) electrons. For projectile energies E_p of the order of 100–500 keV, H_R is given by the relation

$$H_R = \eta'_R (\alpha Z)^2 \frac{1}{2E_p \ln(2E_p)}$$

where α is the fine structure constant ($\alpha^{-1} \sim 137$), Z the atomic number of the heaviest atom in the asymmetric environment of the molecule and η'_R is a molecular asymmetry factor which takes into account the effect of molecular structure on H_R . See the accompanying article for a complete discussion of H_R . We note here that at $E_p \sim 100$ keV and $Z = 6$, H_R lies in the range $10^{-11} < H_R < 10^{-10}$. The uncertainty in H_R is primarily a result of the uncertainties of the molecular wave functions used in its calculation. Thus even if we take $h(e^-) = 1$ and assume that it does not decrease as the β slows down, (in fact $h(e^-) \rightarrow 0$ as the β slows down due to velocity dispersion caused by scattering⁹), the largest resulting value of A_R ($A_R < 10^{-10}$) would still be much too small to be observed in a direct radiolysis experiment. Current techniques limit such experiments to detection of $A_R > 10^{-3}$.

Larger effects, though less directly related to $h(\vec{r})$, may in fact be obtained by performing scattering experiments using unpolarized electron beams whose energy is of the order of the molecular ionization potential (I). A phenomenological analysis of this possibility has been presented¹⁰ and quantitative estimates of the effects to be expected (an induced helicity between 10^{-3} and 10^{-5} in an initially unpolarized electron beam) have been made²⁵. These estimates show that observation of the helicity is not possible using available techniques, a result corroborated by the preliminary experiments sensitive to an induced helicity of order 10^{-2} which have not seen an effect¹¹.

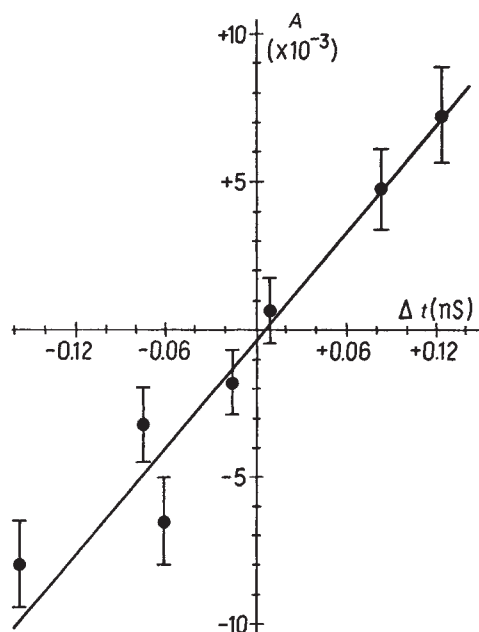


Fig. 3 The observed asymmetry in triplet Ps formation on DL cystine plotted against Δt , the observed time difference between the prompt peaks after helicity reversal. Values of r^+ and r^- based on $\sim 16 \times 10^6$ prompt time events and 10^6 delayed events were used to calculate A_{Ps} , according to equation (4). The uncertainties in A_{Ps} for a 12-h sequence were typically of order 10^{-3} based on Poisson ($\sqrt{N}$) statistics for prompt and delayed counts. The straight line is the linear least squares fit.

Table 1 The residual asymmetry, $A_{Ps}(0)$, and the χ^2 per degree of freedom obtained from the least squares fitting procedure

Substance	D	L	DL
Cystine	$A_{Ps}(0) (\times 10^{-4})$ χ^2/N	-10.8 (5.1) 4.8/5	-3.6 (5.0) 7.4/7
Leucine	$A_{Ps}(0) (\times 10^{-4})$ χ^2/N	-15.1 (7.3) 4.4/5	+4.0 (6.6) 7.3/8
Tryptophan	$A_{Ps}(0) (\times 10^{-4})$ χ^2/N	-2.7 (4.6) 0.7/3	-1.3 (5.5) 3.9/4
			-4.2 (4.9) 6.2/7 -3.3 (3.2) 7.1/6 -10.9 (3.1) 7.0/7

In our experiment we employ a method that is much more sensitive to $h(\vec{r})$ than the radiolysis experiments. A beam of 200–400 eV positrons with a net helicity, $h(e^+)$, is directed into an amino acid target. After slowing down to ~ 10 eV, 80% of the positrons annihilate directly with an electron. The remaining 20% capture an electron to form positronium (Ps), the hydrogen-like bound state of the two particles. The Ps is formed in both the triplet (140 ns lifetime) and singlet (0.1 ns lifetime) spin states and the experiment is designed to detect only the triplet state (based on its long lifetime). Because of the existence of $h(\vec{r})$ in the molecule, the fraction of this state formed for a given isomer $f(\text{L})$ or $f(\text{D})$ depends on the sign and magnitude of $h(e^+)$ that is we have $f^+(\text{L})$, $f^+(\text{D})$ for $h(e^+) > 0$ and $f^-(\text{L})$, $f^-(\text{D})$ for $h(e^+) < 0$. As in radiolysis, $f^+(\text{D}) = f^-(\text{L})$. The asymmetry A_{Ps} in the triplet Ps formation fraction on either reversal of $h(e^+)$ ($h(e^+) \rightarrow -h(e^+)$) or interchange of L and D isomers is:

$$A_{Ps} = \frac{f^+(\text{L}) - f^-(\text{L})}{f^+(\text{L}) + f^-(\text{L})} = \frac{f^+(\text{L}) - f^+(\text{D})}{f^+(\text{L}) + f^+(\text{D})} = |h(e^+)| H_{Ps}(Z) \quad (2)$$

Symmetric expressions for A_{Ps} based on interchange of $f^+(\text{L}) \rightarrow f^-(\text{D})$ and so on may also be written. Here $H_{Ps}(Z)$ is directly analogous to $H_R(E, Z)$, but without the E dependence since Ps formation in a solid occurs in a narrow energy range below the molecular ionization potential. A detailed calculation of $H_{Ps}(Z)$ (ref. 3) shows that in our experimental conditions $H_{Ps}(Z)$ is expected to satisfy the inequality $10^{-6} < H_{Ps}(Z) < 10^{-5}$, for $Z = 6$, although in atypical cases values as large as $H_{Ps}(Z) \sim 10^{-2}$ have not been ruled out. As in the calculation of H_R , the major uncertainty in $H_{Ps}(Z)$ arises from uncertainty in the molecular wave functions.

An approximate general expression³ relating H_R to H_{Ps} may be written as

$$H_R(E, Z) = H_{Ps}(Z) \left(\frac{\eta'_R}{\eta_{Ps}} \right) \left(\frac{1}{2E_p \ln(2E_p)} \right) \quad (3)$$

where η_{Ps} is a molecular asymmetry factor for $H_{Ps}(Z)$, analogous to η'_R for radiolysis. The value of η_{Ps} has been calculated for the simplest optically active molecule for which the most complete wavefunctions are available, 15° twisted ethylene³, with the result $\eta_{Ps} \sim 10^{-2}$. While η'_R has not been so calculated, it is estimated to be of the same order of magnitude as η_{Ps} (although the relative signs are not yet determined). Thus we find that $|H_R| \approx 10^{-5} |H_{Ps}(Z)|$. Our goal is to measure A_{Ps} and $h(e^+)$ and thus determine or set upper limits on $H_{Ps}(Z)$ so that the quantities of fundamental chemical and biological interest, $h(\vec{r})$ and $H_R(E, Z)$, may be estimated.

Experiment

The apparatus is designed to measure the amount of triplet Ps formed when an amino acid powder target is bombarded with a collimated beam of positrons whose energy and helicity can be varied. The asymmetry in triplet Ps production (A_{Ps}) under reversal of either positron helicity (+, -) or target chirality (L, D) is then determined. The apparatus used to produce the beam is sketched in Fig. 1 and discussed in detail in ref. 12.

Figure 1 shows the positrons focused onto the surface of a channel electron multiplier (CEM) coated with the optically active material under investigation, where their impact is detected through emission of secondary electrons (see Fig. 2). Detection of one or more of the annihilation γ s in Pilot B plastic scintillator detectors (Fig. 1) provides the 'stop' signal following

the CEM 'start' signal which allows the lifetime of each positron event to be measured directly. The lifetime spectrum so obtained consists of an ~ 1 ns wide 'prompt peak' of directly annihilating positrons together with Ps which annihilates within the amino acid, the 140 ns exponential component of free triplet Ps, and a uniform background from uncorrelated events. To correct for variations in beam intensity, a ratio of counts from triplet events (background subtracted), occurring in a 400 ns wide time window starting at 75 ns after the prompt peak ($t = 0$), to counts in the prompt peak is calculated. Defining r^+ to be this ratio for incident positron helicity positive (r^- for $\langle h(e^+) \rangle < 0$) A_{Ps} (equation (2)) is given by

$$A_{Ps} = (r^+ - r^-)/(r^+ + r^-). \quad (4)$$

Asymmetry measurements were made by reversing $h(e^+)$ every 330 s during a 12-h period. The cumulative lifetime spectrum for each value of $h(e^+)$ was stored in memory halves of a multichannel analyser. The large number of helicity reversals per run reduced the effect of random and secular time shifts in the $t = 0$ position of the prompt peak to < 0.01 ns. Asymmetries were measured in this manner for samples of D, L, and DL isomers of tryptophan, cystine and leucine (supplied by Sigma Chemical Co.).

Variations in the measured asymmetries for preliminary runs were found to be greatly in excess of statistical fluctuations. Experiments showed that the Ps formation fraction could vary by as much as 20% over the surface of the CEM cone, depending on how far from the channel entrance the positron strikes the cone (see Fig. 2). As a result, changes in the size, shape, and position of the positron beam under helicity reversal could produce false asymmetries as large as 1%. Such systematic effects could be separated from any true isomeric asymmetry by noting that the time-of-flight of the secondary electrons ejected from the cone (and hence the observed time difference, $\Delta\tau$, in the prompt peak after helicity reversal), has also been shown to depend on how far from the channel entrance the beam strikes the CEM. Thus the observed asymmetries A_{Ps} with their correlated values of $\Delta\tau$ could be fit to the equation $A_{Ps} = m\Delta\tau + A_{Ps}(0)$ where m and $A_{Ps}(0)$ are the fitted parameters; and $A_{Ps}(0)$, the residual asymmetry at $\Delta\tau = 0$, is the quantity of interest. An example of the fit is shown in Fig. 3 and the results for the isomers of cystine, tryptophan and leucine are shown in Table 1.

The uncertainties in the results are statistical uncertainties for the fitted data. As evidence by the χ^2 test the linear hypothesis is statistically compatible with the data for the D, L and DL samples of cystine and tryptophan. However, using the original statistically assigned uncertainty, the data of D and L leucine yielded χ^2/N in excess of 2. In view of the still incompletely understood nature of the systematic effects discussed above, we increased the uncertainty on the individual data points to make $\chi^2/N \approx 1$. The uncertainty in $A_{Ps}(0)$ and χ^2/N (leucine) in Table 1 reflect this adjustment.

The measured values of $A_{Ps}(0)$ (Table 1) show no statistically significant difference between the D and L isomers of cystine and tryptophan at the 7×10^{-4} level but the data do indicate the possibility of an effect at the 2σ level in leucine. In view of this possible effect additional data on leucine were obtained in which an attempt was made to maintain $\Delta\tau$ at a sufficiently low level ($\Delta\tau < (0.02 \pm 0.02)$ ns) so that the values of A_{Ps} from several independent runs could simply be averaged without relying on the fitting procedure described above. This was accomplished by adjusting the beam deflection electrodes before each run. The weighted averages and values of χ^2 per degree of freedom obtained using this procedure were $A_{Ps}(D) = -(30.0 \pm 4.9) \times 10^{-4}$, $A_{Ps}(L) = +(1.3 \pm 4.4) \times 10^{-4}$, $\chi^2(D)/N = 5.6/6$ and $\chi^2(L)/N = 11/7$.

The above result seems to indicate the detection of an L/D asymmetry in triplet Ps formation in leucine of $(31 \pm 7) \times 10^{-4}$. However, in view of the large systematic effect discussed previously (values of A_{Ps} of up to -60×10^{-4} were observed in leucine for $\Delta\tau \sim 0.1$ ns) and in view of the fact that $A_{Ps} \sim$

30×10^{-4} is a much larger effect than predicted by theory³, we feel that this result is still preliminary, and while suggestive of an effect, it is not definitive. A newly designed experiment is under construction in which the systematic effects related to beam parameters and detector geometry should be eliminated. We conclude that we have established A_{Ps} to be $< 7 \times 10^{-4}$ in cystine and tryptophan, but that there may be an effect in leucine. We note that previous work by Bonner *et al.*⁴ did show asymmetric L/D radiolysis by polarized electrons in leucine but that this effect was not corroborated by Hodge *et al.*⁸.

To relate A_{Ps} to $H_{Ps}(Z)$ (equation (2)) it is necessary to obtain the positron helicity ($h(e^+) = \langle \hat{s}_i \rangle \cdot \langle \hat{p}_i \rangle$) not for the incident positron beam (recall $h_0(e^+) = 0.21$), but for the beam after it has slowed down in the target to Ps formation energies (≤ 10 eV). This final helicity ($h_f(e^+)$) is not directly observable but it can be estimated theoretically. Many experiments^{12,13} have shown that, in agreement with theoretical predictions¹⁴, the magnitude and direction of $\langle \hat{s} \rangle$ for an ensemble of positrons is essentially unchanged when the positrons slow down to Ps formation energies from energies far in excess of the initial 400 eV energy used in this experiment. Although $\langle \hat{s}_i \rangle$ is constant, the magnitude of $\langle \hat{p}_i \rangle$ ($|\langle \hat{p}_i \rangle|$) does decrease as the beam slows down and this increased beam divergence can be calculated for positrons of the energies we use incident on metal surfaces^{15,16}. For the case of $|\langle \hat{p}_i \rangle|_{\text{initial}} = 1$, the beam divergence after n collisions is given by $|\langle \hat{p}_i \rangle| = \cos \theta = [\cos \theta_1 \cos \theta_2 \cdots \cos \theta_n]$. The theoretical results are generally in good qualitative agreement with experiment^{17,18}.

The calculation of $\cos \bar{\theta}$ using the formulas of refs 17, 18 may be generalized to the case of the amorphous insulator alumina (Al_2O_3) based on calculations of specific energy loss (dE/dx) and mean free path between collisions (λ)¹⁹. The final extrapolation to the amino acids used in our experiment is made based on the assumption that dE/dx and λ may be scaled from one substance to another because the probability of a positron-atom interaction is proportional to $N\sigma$ where N is the number of electrons/atom with which the positron can interact (essentially valence electrons) and σ is the positron-atomic electron interaction cross-section (taken as constant in all amorphous insulators).

The results of the calculation yield a final value of $\cos \bar{\theta} = (0.4 \pm 0.2)$ or $h_f(e^+) = h_0(e^+)|\langle \hat{p}_i \rangle| \approx 0.2 \times 0.4 = (0.08 \pm 0.04)$. The error assignment results from systematic uncertainties in the correct number of valence electrons to use in a given amino acid and the nature of various approximations of the scattering cross-sections used in ref. 19.

Conclusion

Our experiment aims to determine $H_{Ps}(Z)$, or set limits on it, so that the quantity of interest relating to the origin of optical activity $H_R(E, Z)$ may be determined (equation (3)), and so that the newly identified property of chiral molecules, the helicity density $h(\vec{r})$, may be investigated. From equation (2) and the value $h_f(e^+) \approx 0.08 \pm 0.04$ we have $H_{Ps}(Z) \leq (A_{Ps}/h(e^+)) = (7 \times 10^{-4}/8 \times 10^{-2}) \approx 10^{-2}$ for cystine and tryptophan. The possible value of $H_{Ps}(Z)$ for leucine where an effect of $A_{Ps} = (31 \pm 7) \times 10^{-4}$ may have been observed is $H_{Ps}(Z) \approx (31 \times 10^{-4}/8 \times 10^{-2}) \sim (4 \pm 2) \times 10^{-2}$.

Comparing the above results with previous measurements²⁰⁻²³ using positrons we note that, in all cases, positrons from a radioactive source slowed to positronium formation energies directly in the amino acid, implying⁹ $h_f(e^+) < 10^{-2}$. Three experiments²⁰⁻²² gave null values of A_{Ps} at the 10^{-2} level, which if combined with the estimated $h_f(e^+)$, yield $H_{Ps}(Z) < 10$. The positive results of ref. 23 imply $H_{Ps}(Z) \geq 100$. Thus the results of the present experiment, implying $H_{Ps}(Z) \leq 10^{-2}$ in three cases and $H_{Ps}(Z) = 4 \times 10^{-2}$ in the fourth, although larger than the theoretical predictions of ref. 3 ($H_{Ps}(Z) < 10^{-5}$), are the first to set limits on $H_{Ps}(Z)$ which have any reasonable physical meaning, that is $H_{Ps}(Z) < 1$. Finally, using equation (3), our limits on $H_{Ps}(Z)$ enable us to set the upper limit

$H_R(E, Z) < 10^{-7}$. This represents an improvement of 10^4 over the limits of 10^{-3} set by direct radiolysis experiments.

Plans for future experiments involve changes to reduce the instrumental asymmetries and to increase the beam rate by an order of magnitude. In addition, we are now able to increase beam polarization up to 0.7 (J.V.H. and P.W.Z. in preparation). These and other changes should enable us to improve our limits on H_{Ps} by a further factor of ~ 100 , sufficient to observe an effect if $H_{Ps}(Z) \sim 10^{-4}$, a value within theoretical

bounds. Finally, the predicted³ increase in asymmetry with Z^2 will be tested using amino acids with heavy atoms ($Z > 30$) at the chromophore. Observation of the asymmetry in such a molecule should be within reach of the next generation experiment.

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β Decay and the origins of biological chirality: theoretical results

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A dynamical mechanism is found whereby a dissymmetric molecule and its mirror image are ionized at different rates by longitudinally polarized electrons such as produced by nuclear β decay. An enhancement is predicted for molecules containing heavy atoms. Order-of-magnitude estimates indicate that the asymmetric effect of this mechanism may be detectable by current experiments on positronium formation.

THE postulated existence of a causal relationship between the parity nonconserving aspect of the weak interaction and the observed dissymmetry of the molecules present in living organisms^{1–3} seems reasonable as the weak interaction is universal and always acts in the same chiral sense. Hence if an effective mechanism exists involving the weak interaction in molecular evolution, this inherent universal chirality in nature could have influenced the selection of exclusively D sugars for RNA and DNA and L amino acids for proteins.

Two candidates for such a mechanism have been proposed, each of which involves a different aspect of the weak interaction. One produces an energy difference between a chiral molecule and its mirror image⁴, but the calculated difference⁵ seems too small to have been effective in molecular evolution. The other, which seems the most popular theory at present, postulates the asymmetric radiolysis of racemic mixtures of prebiotic chiral molecules by the longitudinally polarized electrons produced in nuclear β decay⁶. A precise test of some aspects of this mechanism is the object of new experiments to measure an asymmetry in the rate of triplet positronium (Ps) formation in chiral molecules discussed in the accompanying article⁷ (where references to previous work may be found).

The present article gives a theoretical basis for, and estimates the magnitudes of, the cross-section asymmetries for both Ps formation and radiolysis. The relevance of the results to the question of the origin of chirality in living organisms is discussed extensively in the preceding article⁷.

Ps formation in optically active molecules

The treatment of the asymmetry in the cross-section for positronium formation given here and that for radiolysis which follows are based on nonrelativistic scattering theory. The notation of Taylor⁷ is followed except that atomic units are used here ($\hbar/2\pi = e = m_e = 1$, where $\hbar$ is Planck's constant, e the electron charge, and m_e the electron mass).

Consider a positron with momentum $\vec{p}$ and helicity $\lambda = \vec{\sigma} \cdot \hat{p} = \pm 1$, where $\hat{p} = \vec{p}/p$ and $p = |\vec{p}|$, incident on an optically active molecule, the nuclei of which are taken to be fixed in the laboratory frame of reference (Fig. 1). This initial channel is labelled α , and α' labels the final channel which consists of the ionized molecule and a Ps atom with momentum $\vec{P}'$. Spin is quantized along $\vec{p}$ and hence the spin projection quantum number of the positron is related simply to the helicity by $m_\lambda = \frac{1}{2}\lambda$. The cross-section depends on the helicity λ , the chirality of the target molecule (L or D), and on the spin state S of the Ps, and is given by

$$\sigma_S^\lambda(L) = (2\pi)^4 \frac{P'}{p} \int |t_S^\lambda(L)|^2_{Av} d\Omega' \quad (1)$$

(with a corresponding expression for the D isomer) where $P' = |\vec{P}'|$, where $d\Omega'$ denotes an integration over the solid angles corresponding to directions of $\vec{P}'$, and where Av denotes an average over all orientations of the molecule (random orientations are assumed). The quantity t_S^λ , which is related to the on-shell T-matrix, is defined below.

In a notation consistent with that of the preceding paper⁷, the asymmetry in the cross-section is defined as

$$H_{Ps}(L) \equiv \frac{\sigma_S^+(L) - \sigma_S^-(L)}{\sigma_S^+(L) + \sigma_S^-(L)} = \frac{\sigma_S^+(L) - \sigma_S^+(D)}{\sigma_S^+(L) + \sigma_S^+(D)} \quad (2)$$

(with a corresponding equation for the D isomer) where $\sigma_S^+(L)$ and $\sigma_S^-(L)$ are obtained from equation (1) with $\lambda = +1$ and $\lambda = -1$ respectively. The second equation in equation (2) is a consequence of parity conservation for the electromagnetic interaction [equations (6, 7)], from which the general relationship $\sigma_S^\lambda(L) = \sigma_S^{-\lambda}(D)$ follows. For the case of a positron beam of helicity $h(e^+)$ and with $h = |h(e^+)| \leq 1$, it can be shown that, for a given L or D isomer (from now on the isomer is not denoted explicitly), the asymmetry in the cross-section obtained

by reversing the beam helicity is

$$A_{Ps} = \frac{\sigma_s^{+h} - \sigma_s^{-h}}{\sigma_s^{+h} + \sigma_s^{-h}} = hH_{Ps} \quad (3)$$

The dynamics which produce the cross-section asymmetry are now considered. For simplicity, a single-electron treatment of the molecular bound state is given here and illustrated in Fig. 1. The positron, 'a', is incident on a molecule consisting of an electron 'b' bound to a core 'c'. The core approximates the fixed nuclear framework and the other electrons in the molecule and provides an effective potential for b. The spin of b, like that of a, is quantized along $\hat{p}$ with its direction given by $s \equiv 2m_s = \pm 1$, where m_s is the spin projection quantum number. The electron bound state is denoted ψ_s .

The cross-section is given by equation (1), where, for a spin-unpolarized target molecule

$$|t_s^\lambda|^2 = \frac{1}{2} \sum_{m_s} \sum_{M_s} |t_{SM_s}^{m_\lambda m_s}|^2 \quad (4)$$

where the second summation is taken over the spin projection quantum number M_s corresponding to the spin S . The on-shell T-matrix is given by

$$t_{SM_s}^{m_\lambda m_s} = \langle \vec{p}' \phi_{Ps} SM_s - |V^\alpha| \vec{p} m_\lambda \psi_s \rangle \quad (5)$$

where ϕ_{Ps} denotes the spatial part of the Ps wave function, V^α denotes the potential energy giving the interaction between the incident positron and the molecule, and the minus sign denotes the prior form⁸ of the T-matrix.

It can be shown on the basis of equations (1)–(5) that H_{Ps} vanishes unless (1) the molecule is dissymmetric and (2) spin-dependent interactions are involved. According to equation (5) there are three possible ways for the effects of spin-dependent forces to occur: (1) in the interaction potential V^α , (2) in the positronium wave function, and (3) in the molecular wave function ψ_s . An investigation of each of these possibilities indicates that for molecules containing atoms as large as carbon, or larger, the dominant contribution to the asymmetry H_{Ps} is expected to come from the perturbation of the electron bound state ψ_s by the spin-orbit interaction of the bound electron moving in the electric field of the molecular core. In what follows, only this bound-state spin-orbit interaction is kept and all other spin-dependent terms in the hamiltonian are ignored. The resulting description is called the bound helical electron model, because, as will be seen later the theory predicts the existence of a helicity density for the electrons bound in an optically active molecule. The qualitative aspects of this model resemble the "helical electron gas" model of Hrasko and Garay⁹.

The dynamics of the bound helical electron model are expressed in the total Hamiltonian $H = H^\alpha + V^\alpha$ for the system, where the channel α Hamiltonian is given by

$$H^\alpha = T_a + T_b + V_{bc}^{\text{COUL}} + V_{bc}^{\text{SO}} \quad (6)$$

and the interaction V^α by

$$V^\alpha = V_{ab}^{\text{COUL}} + V_{ac}^{\text{COUL}} \quad (7)$$

where the subscripts on the kinetic energy operators T and potential energy operators V denote the particles a, b and core c, and where 'COUL' and 'SO' denote the Coulomb and spin-

orbit interactions, respectively. Now the asymptotic stationary state $|\vec{p} m_\lambda \psi_s\rangle$ appearing in equation (5) is an eigenfunction of H^α with eigenvalue E , the total energy of the system, so that when the spin-orbit energy is small compared with E first-order perturbation theory may be used to obtain the result

$$|\psi_s\rangle = \left(\phi_0 + s \sum_n \varepsilon_n^0 \phi_n \right) |m_s\rangle + \sum_n \varepsilon_n^s \phi_n |\bar{m}_s\rangle \quad (8)$$

where $\phi_0|m_s\rangle$ is the unperturbed molecular bound state, $\phi_n|m_s\rangle$ are excited states, $\bar{m}_s = -m_s$, and

$$V_{bc}^{\text{SO}} = \vec{A}_{bc} \cdot \vec{\sigma}_b \equiv \frac{1}{2} \alpha^2 \vec{E}_{bc} \times \vec{p}_b \cdot \vec{\sigma}_b \quad (9a)$$

$$\vec{E}_n = (E_0 - E_n)^{-1} \langle \phi_n | \vec{A}_{bc} | \phi_0 \rangle \quad (9b)$$

$$\varepsilon_n^0 = \vec{E}_n \cdot \hat{e}_3 = \vec{E}_n \cdot \hat{p} \quad (9c)$$

$$\varepsilon_n^\pm = \vec{E}_n \cdot \hat{e}_\pm = \vec{E}_n \cdot (\hat{e}_1 \pm i\hat{e}_2) \quad (9d)$$

Here $\alpha \sim 137^{-1}$ is the fine structure constant, $\vec{E}_{bc}$ is the electric field produced by the core c at the location of electron b, $\vec{p}_b$ is the momentum of b, $\vec{\sigma}_b$ is the Pauli spin matrix, and $\hat{e}_k$ are unit vectors with $\hat{e}_3 = \hat{p}$.

The asymmetry of the positronium formation cross-section is now considered within the bound helical electron model. It can be shown that the 'spin-flip' term in the perturbed molecular wave function, that is, the last term of equation (8), does not contribute to the cross-section to first-order in the spin-orbit interaction and hence can be ignored. The contributing part of equation (8) is now abbreviated $\phi_s|m_s\rangle$, where

$$\phi_s = \phi_0 + s \sum_n \varepsilon_n^0 \phi_n \quad (10)$$

Substitution of $\phi_s|m_s\rangle$ for $|\psi_s\rangle$ in equation (5) then gives

$$t_{SM_s}^{m_\lambda m_s} = V^s \langle SM_s | m_\lambda m_s \rangle \quad (11)$$

where

$$V^s = \langle \vec{p}' \phi_{Ps} - |V^\alpha| \vec{p} \phi_s \rangle \quad (12)$$

The spin-dependent factors appearing in equation (11) are easily evaluated to obtain the contributing T-matrix elements in terms of V^s , and then equations (1)–(4) are used to obtain the cross-section asymmetry H_{Ps} or A_{Ps} to first order in the spin-orbit interaction. For the triplet state the result is

$$H_{Ps} = \frac{1}{6} \frac{\int \{ |V^+|^2_{\Delta v} - |V^-|^2_{\Delta v} \} d\Omega'}{\int |V^0|^2_{\Delta v} d\Omega'} \quad (13)$$

where $V^\pm$ are defined by equation (12) and where V^0 is obtained by replacing ϕ_s by ϕ_0 on the right-hand side of equation (12). The result for the singlet state is obtained by replacing the factor $1/6$ in equation (13) by $-1/2$.

The precise calculation of H_{Ps} is an extremely difficult collision problem. Its order of magnitude can be estimated simply, however, using equations (9)–(13), from which it follows that H_{Ps} is first order in the spin-orbit coefficient ε_n . On the basis of dimensional arguments H_{Ps} would then be expected to be of order $|\varepsilon_n| \sim (\alpha Z)^2 = (Z/137)^2$ where Z is the electric charge on the heaviest atom or atoms in any asymmetric environment in the molecule⁴. Following the treatment of the parity nonconserving energy difference⁴, H_{Ps} is expressed

$$H_{Ps} = \eta_{Ps} (\alpha Z)^2 \quad (14)$$

where the molecular asymmetry factor η_{Ps} is included to take into account the effect of the molecular environment on the magnitude of H_{Ps} . An approximate calculation of H_{Ps} (R.H. and R. Stewart, unpublished data; see also ref. 5) for the simplest dissymmetric molecules for which a wave function has been published, namely twisted ethylene, suggests $\eta_{Ps} \sim 10^{-2}$ to 10^{-3} , which may be typical, although values outside this range cannot be ruled out until more extensive calculations have been performed.

At present the Michigan experiments⁷ have examined three amino acids, two of which give the result $|H_{Ps}| < 10^{-2}$ in agreement with the present theoretical predictions (taking $\eta_{Ps} = 10^{-2}$

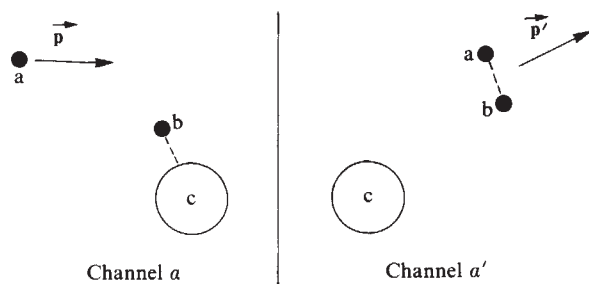


Fig. 1 The formation of positronium.

to 10^{-3} gives $H_{Ps} \sim 10^{-5}$ – 10^{-6} for amino acids). The third amino acid, leucine, gives the preliminary result $H_{Ps} = (4 \pm 2) \times 10^{-2}$ in apparent disagreement with theory. Although it is conceivable that the present theory could obtain such a large value of H_{Ps} in atypical cases, it is premature to attempt to make a more detailed analysis for the case of leucine at present due to the difficulty of even crude calculations involving amino acid wave functions and also due to the preliminary nature of the experimental result.

Finally, in view of the treatment of radiolysis to be given next, it should be noted that the asymmetry in the total inclusive cross-section for Ps formation, obtained by summing equation (1) over $S = 0$ and 1, vanishes identically due to the cancellation of the singlet and triplet contributions. A vanishing result is also obtained for ionization without Ps formation, which is closely related to the process of ionization by polarized electrons treated next.

Electron impact ionization of optically active molecules (radiolysis)

The theoretical treatment of radiolysis is similar to that just given for Ps formation. The most important difference is that, because the electrons are identical, the two-electron state of incident plus target electron is antisymmetric with respect to the interchange of these particles, and as a consequence both direct and exchange terms appear. In fact it is the existence of these exchange terms which leads to a nonvanishing asymmetry in the total cross-section, as is shown below, in contrast to the case of ionization by positrons discussed above.

The scattering process is illustrated in Fig. 2. In the initial channel α the incident electron 1 has helicity $\lambda = \pm 1$ and momentum $\vec{p}$ and the target electron 2 is bound to the core c . In the two final channels α' and $\tilde{\alpha}'$, which differ by an exchange of the two electrons, the momenta are designated $\vec{p}_a$ and $\vec{p}_b$. The spins are again quantized along $\vec{p}$ with initial spin projection quantum numbers m_λ , m_s and final spin quantum numbers S , M_S describing singlet and triplet states. The total ionization cross-section, inclusive of electrons in singlet and triplet final states, is given (for either the D or L isomer) by

$$\sigma^\lambda = \frac{1}{2}(2\pi)^4 \frac{1}{p} \int_0^{E_p-I} dE_b p_a p_b \int d\Omega_a d\Omega_b |\hat{t}^\lambda|^2_{\Lambda v} \quad (15)$$

where $E_p = p^2/2$ is the energy of the incident electron, I is the molecular ionization energy, $E_b = p_b^2/2$, and where

$$|\hat{t}^\lambda|^2 = \frac{1}{2} \sum_{m_s} \sum_S \sum_{M_S} |\hat{t}_{SM_S}^{m_\lambda m_s}|^2. \quad (16)$$

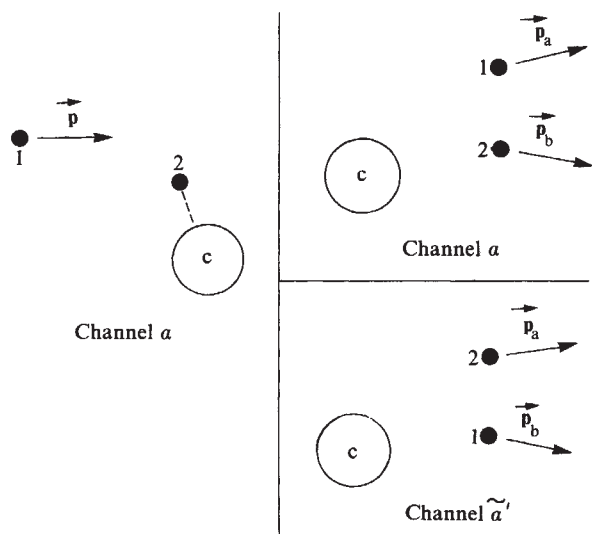


Fig. 2 Ionization by electron impact.

The on-shell T-matrix elements are given by

$$\hat{t}_{SM_S}^{m_\lambda m_s} = t_{SM_S}^{m_\lambda m_s}(\text{dir}) + (-)^S t_{SM_S}^{m_\lambda m_s}(\text{exch}) \quad (17)$$

with the direct and exchange terms given by

$$t_{SM_S}^{m_\lambda m_s}(\text{dir}) = \langle \vec{p}_a \vec{p}_b S M_S - | V^\alpha | \vec{p} m_\lambda \psi_s \rangle \quad (18a)$$

$$t_{SM_S}^{m_\lambda m_s}(\text{exch}) = \langle \vec{p}_b \vec{p}_a S M_S - | V^\alpha | \vec{p} m_\lambda \psi_s \rangle \quad (18b)$$

Equations (18a, b) are analogous to equation (5) and ψ_s is again given by equation (8). The same procedure which led from equation (5) to equation (13) is again followed here using equations (15)–(18) and gives the following result for the asymmetry in the radiolysis cross-section, correct to first order in the spin-orbit interaction:

$$H_R = \frac{\sigma^+ - \sigma^-}{\sigma^+ + \sigma^-} = -\frac{1}{2} \frac{\int_0^{E_p-I} dE_b p_a p_b \int d\Omega_a d\Omega_b \text{Re} \{ V_d^{*+} V_e^+ - V_d^{*-} V_e^- \}_{\Lambda v}}{\int_0^{E_p-I} dE_b p_a p_b \int d\Omega_a d\Omega_b \{ |V_d^0|^2 + |V_e^0|^2 - \text{Re} V_d^{*0} V_e^0 \}_{\Lambda v}} \quad (19)$$

where

$$V_d^s = \langle \vec{p}_a \vec{p}_b - | V^\alpha | \vec{p} \phi_s \rangle \quad (20a)$$

$$V_e^s = \langle \vec{p}_b \vec{p}_a - | V^\alpha | \vec{p} \phi_s \rangle \quad (20b)$$

are direct and exchange integrals with ϕ_s again given by equation (10). The integrals V_d^0 and V_e^0 are obtained by replacing ϕ_s with ϕ_0 in equations (20a, b). If the incident electron state is a mixture of pure helicity states with a net helicity $h(e^-)$, then the asymmetry in the cross-section is

$$A_R = \frac{\sigma^{+h} - \sigma^{-h}}{\sigma^{+h} + \sigma^{-h}} = h H_R \quad (21)$$

where $h = |h(e^-)|$. A negative value of H_R or A_R for a given L or D isomer implies that the isomer is preferentially ionized by natural β electrons, for which $h(e^-)$ is negative.

Although the precise calculation of H_R is difficult, it is possible to make rough estimates of its order of magnitude as was done for H_{Ps} . It is useful to distinguish two cases. For the case of incident energies E_p near the ionization threshold, the final momenta $\vec{p}_a$, $\vec{p}_b$ both approach zero, so it follows from equations (20a, b) that $V_e^s \rightarrow V_d^s$ (note that only scattering into the singlet state contributes in this limit), and then from equation (19) that

$$H_R \xrightarrow{E_p \rightarrow I} \frac{1}{2} \frac{|V_d^{*+}|^2_{\Lambda v} - |V_d^{-}|^2_{\Lambda v}}{|V_d^0|^2_{\Lambda v}} \quad (22)$$

which, on the basis of dimensional arguments similar to those which led to equation (14), can be shown to have the same order of magnitude as H_{Ps} . Hence H_R is expressed

$$H_R = \eta_R (\alpha Z)^2 \quad (E_p \rightarrow I) \quad (23)$$

where the molecular asymmetry factor for radiolysis η_R is expected to have the same order of magnitude as η_{Ps} . For the case of incident energies near 100 keV, which is typical of β -decay electrons, use of the nonrelativistic Bethe approximation¹⁰ in equation (19) gives the result

$$H_R = \frac{\eta'_R (\alpha Z)^2}{(2E_p) \ln(2E_p)} \quad (1 \ll E_p \ll \alpha^{-2}) \quad (24)$$

where η'_R is formally of the order of one but is expected to be less than one for the same reasons given in connection with equations (14) and (23). The range for E_p corresponds to the range of validity for the nonrelativistic Bethe approximation¹⁰; for more precise calculations relativistic effects should be included. For $E_p = 100 \text{ keV} \approx 3,700 \text{ AU}$ equation (24) gives $H_R \approx 10^{-5} \eta'_R (\alpha Z)^2$ which is roughly five orders of magnitude smaller than the threshold value. This large reduction is due primarily to the decreasing magnitudes of the exchange integrals V_e^s with increasing energy. Taking η'_R to be of the order 10^{-2} – 10^{-3} in equation (24) gives an estimated asymmetry $H_R \sim$

10^{-10} – 10^{-11} for the radiolysis of amino acids ($Z=6$) with 100-keV electrons and a magnitude roughly 100 times larger for a molecule containing a dominant heavy atom with Z near 100.

Recently Zel'dovich and Saakyan¹¹ have calculated a contribution to H_R which is not included in equation (19) but rather is due to the direct spin-dependent interaction between the incident and target electrons, and which may be expressed

$$H_R(\text{ref. 10}) \approx \alpha^2 \frac{v}{c} \frac{\text{Im}(\vec{d}_i \cdot \vec{m}_i^*)}{\sum_i |\vec{d}_i|^2} \quad (25)$$

where v/c is the ratio of the speed of the incident electron to the speed of light, and where $\vec{d}_i$ and $\vec{m}_i$ are the electric and magnetic transition moments, respectively, between the initial and final molecular states. The ratio of sums appearing in equation (25) may be estimated from spectral data on optically active molecules¹². For strong absorption bands, which are expected to dominate the cross-section, this ratio is typically of the order of 10^{-6} , which is much less than the estimated value 10^{-2} – 10^{-3} based on dimensional arguments¹¹. Equation (25) then gives the result H_R (ref. 11) $\approx 10^{-11}$ for 100 keV electrons, which is comparable with the result for the bound helical electron model at the same energy and with $Z \approx 6$. Hence the contribution to H_R from equation (19) dominates at low energies and high Z , but the contributions from both equations (19) and (25) are important at high energies.

More recently, Mann and Primakoff¹³ have estimated H_R to be relatively large (of the order of $\alpha^2 \approx 10^{-4}$) due to the interaction between the spin of the incident beta electron and the orbital angular momentum of the target electron. This estimate is in direct disagreement with that from ref. 12. My preliminary calculation gives the result that the contribution to H_R from this spin–other-orbit interaction, although formally of the order of α^2 , vanishes in the first Born approximation when the average over molecular orientations is taken.

I conclude this section with the following remarks: (1) β Electrons are emitted with a spectrum of energies ranging from zero up to a maximum which is typically hundreds of keV. In view of the present results, the low energy end of this spectrum may be more important for asymmetric radiolysis than previously thought, even though the magnitude of the helicity is less at low energies. (2) Experiments which attempt to detect this asymmetry are expected to find a larger effect for low-energy polarized electrons, particularly with dissymmetric target molecules containing heavy atoms.

Electron helicity in bound states

For a one-electron state ψ_s which is characterized by the spin direction $s \equiv 2m_s$ the helicity density is defined in coordinate space by the equation

$$h_s(\vec{x}) = \text{Re}\{\psi_s^\dagger(\vec{x}) \vec{\sigma} \cdot \hat{p}_e \psi_s(\vec{x})\} \quad (26a)$$

or in momentum space by

$$h_s(\vec{p}_e) = \text{Re}\{\psi_s^\dagger(\vec{p}_e) \vec{\sigma} \cdot \hat{p}_e \psi_s(\vec{p}_e)\} \quad (26b)$$

where $\vec{\sigma}$ is the Pauli spin operator, $\vec{p}_e$ is the electron momentum, and $\hat{p}_e = \vec{p}_e/|\vec{p}_e|$. The two-component spin functions $\psi_s(\vec{x})$ and $\psi_s(\vec{p}_e)$ are Fourier transforms of each other. For a one-electron atom or molecule in a pure spin state, the helicity density can be relatively large, of the order of several atomic units.

The helicity density may also be defined in a general way for a many-electron system, but here it is sufficiently general to consider the simple case of a two-electron singlet state

$$\Psi(1, 2) = 2^{-1/2} [\psi_+(1)\psi_-(2) - \psi_-(1)\psi_+(2)] \quad (27)$$

for which the helicity density is given by

$$h(\vec{p}_e) = \sum_{s=\pm 1} \text{Re}\{\psi_s^\dagger(\vec{p}_e) \vec{\sigma} \cdot \hat{p}_e \psi_s(\vec{p}_e)\} \quad (28a)$$

$$= \sum_{s=\pm 1} h_s(\vec{p}_e) \quad (28b)$$

In this case it can be easily shown that, in the absence of spin–orbit coupling, the helicity density vanishes due to cancel-

lation of equal and opposite contributions from the two spin states. If spin–orbit coupling corrections to the wave function $\Psi(1, 2)$ are now included by perturbing the spin orbitals ψ_s according to equation (8), then equation (28) gives

$$h(\vec{p}_e) = 4Re \sum_n \vec{\epsilon}_n \cdot \hat{p}_e \phi_0^*(\vec{p}_e) \phi_n(\vec{p}_e) \quad (29)$$

where $\vec{\epsilon}_n$ is defined in equation (9b). The same result (divided by two) is obtained for a spin-unpolarized one-electron state. The following comments regarding equation (29) can be made: (1) h is formally of the order of $|\epsilon_n| \sim (\alpha Z)^2$ atomic units; (2) h vanishes for an atom or for a molecule with a centre of inversion symmetry; (3) h is in general non-zero for a molecule with a fixed orientation and without a centre of inversion; (4) if an average over orientations is taken for an ensemble of randomly oriented molecules, the resulting average helicity density h_{Av} is independent of the direction of $\vec{p}_e$ and is non-zero only in the case of a dissymmetric molecule; (5) the expectation value $\langle \vec{\sigma} \cdot \hat{p}_e \rangle$, which is obtained from the right-hand side of equation (29) or equation (28a) by replacing $\hat{p}_e$ with $\vec{p}_e$ and integrating over all momentum space, is identically zero to first order in the spin–orbit interaction. Proofs of these statements will be given elsewhere. The helicity density is an observable property of atoms and molecules which apparently has not been considered previously.

The asymmetry in the cross-sections for triplet Ps formation in dissymmetric molecules may be calculated from the helicity density by means of an approximate equation derived from equation (13):

$$H_{Ps} \approx \frac{1}{6} \frac{\int d\Omega' |F_{Ps}(\vec{Q})|^2 \hat{p} \cdot \hat{q} h_{Av}(q)}{\int d\Omega' |F_{Ps}(\vec{Q})|^2 |\phi_0(\vec{q})|_{Av}^2} \quad (30)$$

where $F_{Ps}(\vec{Q})$ is the Fourier transform of the product of the Ps wave function and an effective potential energy for the interaction between the incident and target particles, $\vec{q} = \vec{P}' - \vec{p}$, $\vec{Q} = \frac{1}{2}(\vec{q} - \vec{p})$, and where the other quantities have been defined previously. A similar approximate relation is obtained for the radiolysis asymmetry:

$$H_R \approx - \frac{1}{2} \frac{\int_0^{(E_p-1)/2} dE_b p_a p_b \int d\Omega_a d\Omega_b F_R(\vec{Q}_a) F_R(\vec{Q}_b) \hat{p} \cdot \hat{q} h_{Av}(q)}{\int_0^{(E_p-1)/2} dE_b p_a p_b \int d\Omega_a d\Omega_b [F_R^2(\vec{Q}_a) + F_R^2(\vec{Q}_b) - F_R(\vec{Q}_a) F_R(\vec{Q}_b)] |\phi_0(\vec{q})|_{Av}^2} \quad (31)$$

where F_R is defined similarly to F_{Ps} and where $\vec{Q}_a = \vec{p}_a - \vec{p}$, $\vec{Q}_b = \vec{p}_b - \vec{p}$, and $\vec{q} = \vec{p}_a + \vec{p}_b - \vec{p}$. The effect of F_{Ps} or F_R is to weight the integrands in equations (30) and (31) heavily towards small values of $\vec{Q}$, $\vec{Q}_a$, and $\vec{Q}_b$ to the extent allowed by energy and momentum conservation. The primary advantage of writing H_{Ps} and H_R in terms of the helicity density is a simple unified semiquantitative understanding of the mechanism producing these asymmetries which is reminiscent of the qualitative ‘‘helical electron gas’’ model of Garay and Hrasko⁹.

Equations (30) and (31) indicate that the signs of H_{Ps} and H_R are determined by the function $\hat{p} \cdot \hat{q} h_{Av}(q)$, since all of the other quantities appearing in these equations have a positive sign. In view of the expected difficulty of accurately calculating the sign of h_{Av} theoretically, an important question is whether the sign of H_R at high energies can be predicted from a knowledge of the sign of H_{Ps} at low energies, as the latter may be determined experimentally by the methods of ref. 7. As yet the dependence of h_{Av} on the bound-electron momentum q is not understood sufficiently to answer this question, but if it can eventually be answered in the affirmative, the positronium formation asymmetry measurements may then be able to provide the answer to the important question of the relative susceptibility of D compared with L isomers to ionization by the polarized electrons from nuclear β decay.

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Secretory protein translocation across membranes—the role of the ‘docking protein’

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On emergence of the signal sequence of nascent secretory proteins from the large ribosomal subunit, translation is stopped by a cytoplasmic protein complex. A specific membrane protein of the endoplasmic reticulum, the ‘docking protein’, releases this block and allows further synthesis to be directly coupled to transfer across the membrane.

NASCENT secretory proteins are extended at the N-terminus by 15–30 amino acids, the signal or leader sequence^{1–3}. This extension, or its functional equivalent⁴, is required for the vectorial transfer of the protein across the membrane of the endoplasmic reticulum (ER)^{1,5–9}. On emergence of the signal sequence from the large ribosomal subunit, the ribosomal complex specifically makes contact with the membrane, then the nascent protein is translocated across the ER membrane. As the translocation phenomenon has been reconstituted *in vitro* using isolated rough microsomes^{7,10}, the molecules which mediate this transfer must be present in, and dissectable from, these membranes^{11–16,17}.

The ability to transport nascent secretory proteins is lost when rough microsomes are exposed to high salt concentrations¹⁸. A protein complex of molecular weight (MW) 250,000 (250K) is removed from the membrane in these conditions, which results in the loss of translocation activity^{19,20}. Recently, it was determined that this 250K protein, referred to as the signal recognition protein (SRP), functions *in vitro* by selectively stopping the translation of nascent secretory proteins²¹. When salt-washed rough microsomes are added to the SRP-blocked system, translation continues and translocation of the nascent peptide occurs²¹. The question then arises as to which ER-specific membrane component is responsible for relieving the translation block. We have recently shown that a 60K peptide can be released from salt-washed rough microsomes by elastase and high salt treatment^{15,16}. This component is ER-specific, is required for vectorial transfer of secretory proteins, and is derived from a membrane-bound protein of molecular weight 72K (ref. 22). So far, the role of the 72K protein has remained unknown.

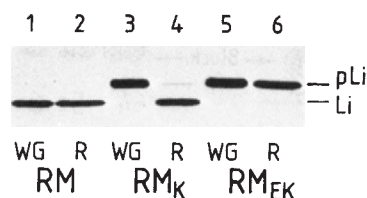


Fig. 1 Translocation activity of rough microsomes assayed in wheat germ and reticulocyte lysate. RM, whole rough microsomes; RM_K, KCl-washed RM; RM_{EK}, elastase-treated, KCl-washed RM; WG, wheat germ lysate; R, reticulocyte lysate system; pLi, pre-IgG light chain; Li, authentic IgG light chain. Membranes were prepared and *in vitro* translations were performed as described previously^{15,18}. Translocation activity is measured as the ability of rough microsomes to convert pLi to Li when added to a cell-free system primed with mRNA encoding mainly IgG light chain. The figure shows a fluorogram depicting the translation products of *in vitro* synthesis.

Table 1 Ability of protein components to restore translocation activity to microsomal membranes

Microsomes/components	Activity in:	
	Wheat germ	Reticulocyte lysate
RM	+	+
RM _K	—	+
RM _K + SRP	+	+
RM _K + DP _f	—	+
RM _{EK}	—	—
RM _{EK} + DP _f	—	+
RM _{EK} + SRP	—	—
RM _{EK} + DP _f + SRP	+	+

Rough microsomes (RM) in combination with isolated factors (DP_f or SRP) were tested for their ability to translocate nascent proteins in wheat germ and reticulocyte lysate systems. To exclude the possibility that reconstitution was being mediated by contaminating proteins in DP_f preparations, controls were done using a specific anti-DP_f antibody²². In these controls the antibody was used to specifically remove DP_f from the preparations. Such controls were performed in every case where DP_f was used in this report, and each time the preparation treated in this way was unable to reconstitute activity in inactive rough microsomes. RM_K, KCl-washed RM; RM_{EK}, elastase-treated and KCl-washed RM; SRP, signal recognition protein; DP_f, cytoplasmic fragment of docking protein released by elastase and high salt. Microsomes and factors were prepared and assays carried out as described previously^{15,16,20}.

We report here that this protein is indeed the molecule which relieves the SRP-induced block. Translation can then proceed when the initiated ribosomal complex has made contact with the correct membrane, that is, the one containing the 72K protein. In accordance with this function, we shall henceforth refer to this molecule as the ‘docking protein’ of the ER. We also report the presence in the cell cytoplasm of a functional equivalent of the microsomal signal recognition protein.

Signal recognition protein interacts with docking protein

Previously published data suggested that the source of the cell-free translation system affected greatly the translocation capability observed for salt-washed microsomes (compare refs 14, 15 with 19, 20). Whole rough microsomes (RM) were fully active in the wheat germ and reticulocyte lysate systems (Fig. 1, lanes 1 and 2). Salt-washed microsomes (RM_K), however, were only active in the reticulocyte lysate (compare Fig. 1, lane 3 with lane 4). Microsomes treated with protease and high salt (RM_{EK}) were inactive in either system (Fig. 1, lanes 5 and 6).

Salt washing exerts its effect by removing the 250K complex (SRP) from rough microsomes^{19,20}, whereas salt washing in conjunction with proteolysis removes from RM_K an active 60K cytoplasmic domain from the 72K docking protein (referred to as 'docking protein fragment', or DP_f)¹⁶. To define the requirements of translocation more precisely, we recombined the two factors with the various inactive rough microsomes and tested these in the two translation systems. The addition of the 60K fragment conferred activity on RM_{EK} in the reticulocyte lysate system alone; this combination is hence functionally equivalent to RM_K (see Table 1). It should then be possible to add SRP to $RM_{EK} + DP_f$ and restore activity in the wheat germ system. This was indeed the case, suggesting a functional identity between $RM_{EK} + DP_f + SRP$ and whole rough microsomes. We therefore conclude that two components are required to restore full activity to RM_{EK} : the 60K DP_f fragment and the 250K complex (SRP). It is clear also that reticulocyte lysate contributes the functional equivalent of the SRP.

The fact that the SRP and the DP_f could be removed sequentially from rough microsomes suggested a possible interaction between them. To test this, membranes (RM_{EK}) were incubated with either SRP or DP_f . After washing, the membranes were assayed in the wheat germ system in the presence of DP_f or SRP, respectively. In agreement with the results given above (Fig. 1), neither RM_{EK} (Fig. 2a, lane 1) nor RM_{EK} charged with

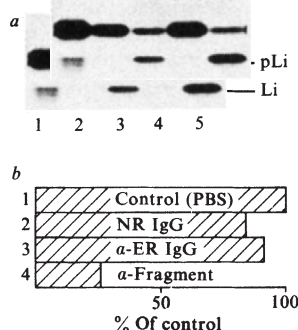


Fig. 2 Sequential reconstitution of translocation activity from isolated components. Inactive rough microsomes (RM_{EK}) were allowed to bind DP_f or SRP, and assayed in wheat germ in the presence of the component that was omitted from the preincubation. *a*, Lane 1, RM_{EK} ; lane 2, RM_{EK} charged with DP_f ; 3, RM_{EK} charged with DP_f assayed in the presence of SRP; 4, RM_{EK} charged with SRP assayed in the presence of DP_f ; 5, intact rough microsome control. RM , DP_f and SRP were prepared as described elsewhere^{15,16,20}. Activity is defined as described in Fig. 1 legend. *b*, RM_K were treated with anti- DP_f IgG, washed and assayed for translocation activity in the presence of SRP in a dithiothreitol-free wheat germ system. Lane 1, activity of RM_K pretreated with PBS; 2, activity of RM_K pretreated with normal rabbit (NR) IgG; 3, activity of RM_K pretreated with an affinity-purified anti-ER glycoprotein antibody specific for antigens on the surface of RM_K vesicles; 4, activity of RM_K pretreated with anti- DP_f IgG²². In the membrane-affinity-based translocation assay, 2.5 μ l rough microsomes ($A_{280} = 60 \text{ ml}^{-1}$) were incubated in the presence of the fraction being tested at 0°C for 30 min in conditions where KCl concentration = 50 mM. The membranes were pelleted by centrifugation at 12,500 g for 20 min, then the rough microsome pellets were washed and suspended in the complete wheat germ cell-free translation system and assayed for translocation activity.

DP_f (lane 2) were active. When the $RM_{EK} - DP_f$ membranes were assayed in the presence of SRP (Fig. 2a, lane 3) the activity was restored to the level of whole rough microsomes (Fig. 2a, lane 5). No reconstitution was observed (Fig. 2a, lane 4) for RM_{EK} pre-incubated with SRP and assayed in the presence of DP_f . These data demonstrate that DP_f must be present on the membrane for SRP to interact functionally with rough microsomes.

Further evidence for the functional interaction of DP_f with SRP is provided by data obtained using a specific anti- DP_f antibody²². When RM_K were charged with this antibody (by the method described in Fig. 2 legend), the membranes were

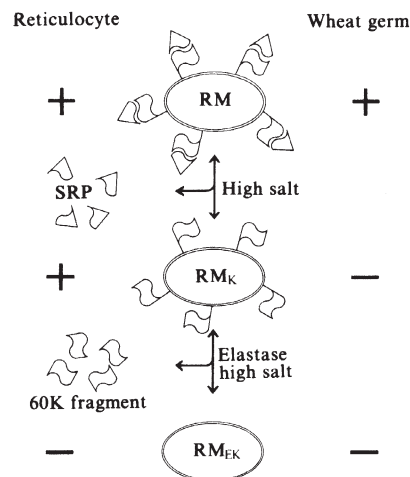


Fig. 3 Dissection and reconstitution of rough microsomes using protease and high salt. When rough microsomes are treated with 0.5 M KCl, translocation activity is lost in wheat germ (due to the salt-mediated removal of SRP) and RM_K are generated which are nonetheless active in reticulocyte lysate. Treatment of salt-washed microsomes (RM_K) with elastase and high salt yields RM_{EK} which are inactive in either system. This is due to the loss of a 60K peptide fragment from the docking protein (DP_f). Reconstitution can be achieved sequentially by re-addition of DP_f to RM_{EK} (thus restoring activity in reticulocyte lysate) followed by re-addition of SRP to $RM_{EK} - DP_f$ membranes. In this way full reconstitution is achieved in both cell-free systems.

no longer able to interact functionally with SRP (Fig. 2b). RM_K incubated with either pre-immune IgG or an ER-specific antibody directed against other antigens on the cytoplasmic surface of rough microsomes were still fully active when assayed in the wheat germ system in the presence of SRP.

A model summarizing these findings is shown in Fig. 3. High salt concentration removes the 250K complex (SRP), yielding RM_K which are inactive in the wheat germ system. Proteolysis and high salt remove the 60K portion of a larger membrane protein (DP), yielding RM_{EK} , which is inactive in either system. Reconstitution occurs in the reticulocyte lysate when DP_f is added to RM_{EK} and in the wheat germ system when DP_f and then SRP are added to RM_{EK} .

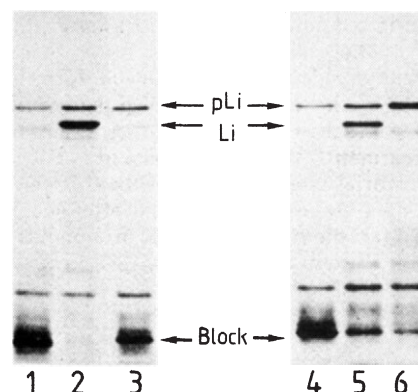


Fig. 4 SRP-induced block of translation of secretory proteins is removed by DP_f . Translations in the wheat germ system were blocked by the addition of SRP. We examined the ability of different membrane preparations to overcome this block. Lane 1, products of light chain mRNA-dependent cell-free translation (15 min) in wheat germ in the presence of SRP. Note the large accumulation of 8–9K material labelled 'block'. Lane 2, addition at 15 min of RM_K to a 'blocked' translation, observed at 40 min. 3, As in lane 2 except that RM_{EK} was added instead of RM_K . 4, Same as lane 1. 5, As in lane 2 except that $RM_{EK} + DP_f$ was added instead of RM_K . 6, As in lane 2 except that DP_f was added instead of RM_K (note the increase in pLi as opposed to Li due to the absence of membranes). Synchronous translation assays were carried out as described by Walter and Blobel²¹.

DP fragment releases SRP-induced translation block

Walter *et al.*²¹ have recently demonstrated that the SRP arrests the translation of preprolactin in the wheat germ cell-free system. This phenomenon is characterized by an accumulation of incomplete nascent peptides having a molecular weight of 7–8K. A similar result was obtained for IgG light chain (Fig. 4, lanes 1 and 4). This block is removed by the addition of RM_K (lane 2). Then the question arises: with which membrane component does SRP interact to result in the resumption of translation? DP_i, RM_{EK} or both could be required. As shown in Fig. 4, the block (lane 1) was removed by adding RM_K (lane 2), but not by addition of RM_{EK} (lane 3). When DP_i was added to the RM_{EK}, the block was removed (lane 5). The same effect was obtained when DP_i alone was added to the blocked system (lane 6). Thus we conclude that DP_i is necessary for the resumption of translation and translocation, while the residual membrane (RM_{EK}) is not.

Translation of secretory proteins blocked by soluble protein in reticulocyte lysate

The ability of RM_K to translocate nascent peptides in the reticulocyte lysate system, but not in wheat germ, suggested that a molecule equivalent to SRP must exist in the reticulocyte lysate. Such a component was characterized by making use of its ability to interact functionally with the membrane in low salt conditions. Lysate fractions were tested by allowing the component to bind to RM_K and assaying for reconstitution of translocation activity in the wheat germ system. The use of such an affinity-based assay was necessary, as direct addition of reticulocyte lysate inhibited the wheat germ cell-free system. Furthermore, it proved extremely sensitive. Several properties of the reticulocyte factor were determined; the results (Table 2) indicate that this factor has several of the basic features of SRP. It is an acidic protein of MW ~250,000, has a hydrophobic character, and blocks translation of nascent secretory proteins. The reticulocyte factor-induced block can be removed by adding RM_K, as was observed in the case of SRP. The presence of a block-inducing substance such as the reticulocyte factor in reticulocyte lysate may explain why the cytoplasmically-located nucleocapsid proteins of certain viral mRNAs were translated well and the membrane proteins only poorly²³. Due to the small quantity of the reticulocyte factor in reticulocyte lysate, it has not been possible to determine whether its subunit composition is the same as that of SRP.

The cytoplasmic location of reticulocyte factor suggested that SRP may also be only transiently associated with the membrane, and that it is present in the cell as a soluble, cytoplasmic factor. Using the membrane-affinity assay described in Fig. 2 legend, we found that post-microsomal supernatants of dog pancreas contained a substance capable of restoring activity to RM_K when assayed in the wheat germ system. It possessed all the

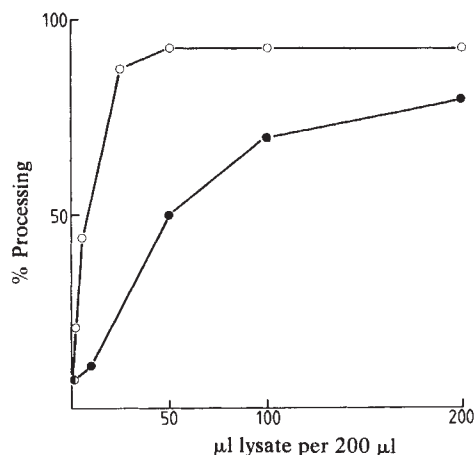


Fig. 5 SRP-like activity of reticulocyte lysate and dog pancreas post-microsomal supernatant. Reticulocyte lysate and dog pancreas supernatants (both depleted of ribosomes by centrifugation) were assayed for their ability to restore translocation activity to RM_K in the wheat germ system. 2.5 μl of RM_K ($A_{280} = 60 \text{ ml}^{-1}$) were incubated with reticulocyte lysate or pancreatic soluble phase (both corrected to 40% (v/v) homogenates) in amounts indicated on the abscissa. The membranes were washed and assayed for translocation activity in wheat germ lysate. Activity is expressed as the % conversion of pLi to Li. ●, Reticulocyte lysate; ○, dog pancreas supernatant.

characteristics of reticulocyte factor (see Table 2) and SRP. In addition, a fivefold higher level of SRP-like activity was found in dog pancreas supernatant when compared with reticulocyte lysate (Fig. 5). These findings are consistent with the functional role of SRP in translocation. The delay in translation of secretory proteins, that is, until contact with the proper membrane has been made, would be optimally localized in the cytoplasmic compartment.

Implications and conclusions

A model consistent with all the data reported here, and with data obtained previously^{14–16,18–22}, is shown in Fig. 6. The initial events in protein translocation can be described as follows. Synthesis of a secretory protein is initiated on free ribosomes. After 60–70 amino acids have been polymerized and the signal sequence has emerged from the large ribosomal subunit, further translation is interrupted by SRP²¹. This block persists until contact is made with the 72K docking protein of which the 60K fragment (DP_i) represents the cytoplasmic domain. At this point translation, coupled with translocation, proceeds.

Such a sequence of events ensures optimal co-translational processing of secretory proteins, even when synthesis commences on free ribosomes. The presence of a cytoplasmic form of

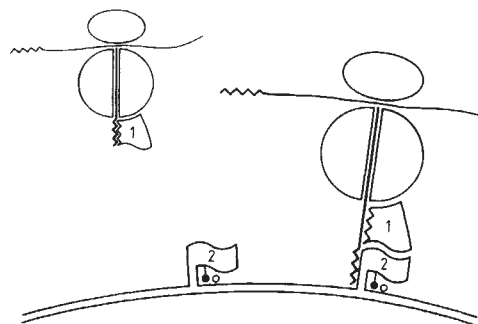


Fig. 6 Sequence of events in ER-specific translocation of secretory proteins. Initiation begins in the cytoplasm on free ribosomes. Translation is blocked by SRP (component 1) after 70–80 amino acids have been polymerized and the signal sequence emerges from the large ribosomal subunit (shown here in cross-section). This arrest of translation persists until contact is made with the 'docking' protein (component 2) which is a 72K, ER-specific membrane protein. Translation then resumes and translocation proceeds.

Table 2 Properties of the reticulocyte factor

Property	Method of determination
Proteinaceous	Proteolysis
Salt-linked to rough microsomes	Microassay
Acidic pI	DEAE binding*
Hydrophobic	ω -NH ₂ pentyl agarose*
MW = 250,000	Sucrose gradient*
Blocks translation of secretory proteins	Translation in wheat germ†

The ability to reconstitute translocation activity of RM_K in the wheat germ system was tested by adding various lysate-derived fractions using the method described in Fig. 2 legend. For proteolysis, the reticulocyte lysate was treated with trypsin ($20 \mu\text{g ml}^{-1}$) for 20 min at 25 °C and then tested in the assay system described in Fig. 2 legend. In the microassay, the reticulocyte lysate was brought to 0.5 M in KCl and tested as above. * Ref. 20. † Ref. 21.

the signal recognition protein which blocks translation would guarantee that pre-secretory proteins are not completed in the cytoplasm. Efficient transfer of proteins can occur, therefore, only after the correct membrane has been contacted. Such a membrane can be operationally defined as one which contains the docking protein. This protein has been shown to be restricted to the endoplasmic reticulum²².

The 72K docking protein is the first site of interaction between nascent secretory proteins and the ER membrane. An understanding of the function of this protein and SRP explains

the specificity but not the mode of translocation. To determine the actual mechanism whereby proteins cross the membrane, further dissection of the membrane, including disassembly of the lipid bilayer, is necessary¹⁷. It is also conceivable that other soluble components provided by the cell-free translation systems function in this process. Their identification would require further fractionation of the lysates.

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Inducibility of human β -interferon gene in mouse L-cell clones

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Transfer of a 36-kilobase piece of human DNA containing the β -interferon (IFN- β) gene into mouse Ltk⁻ cells leads to transient expression of human interferon even without an exogenous inducer. A low level of human interferon expression is also found in most stable clones containing the transferred DNA. With double-stranded RNA or Newcastle disease virus (NDV) as inducer, human interferon expression is greatly increased. The induced transcript is identical to normal human IFN- β mRNA. Neighbouring genes contained on the transferred DNA are co-induced but are not essential for the production of human interferon in mouse L cells.

THE interferons (IFNs) are a class of proteins capable of inducing an antiviral state in many cell types against a wide range of viruses¹. Almost all cell types produce one or more interferons upon induction with virus or a synthetic inducer such as double-stranded RNA¹. FS4 cells, primary human fibroblasts, produce IFN- β (ref. 2) and several other proteins of unknown biological function³ in response to the inducer poly(rI)poly(rC). In these cells interferon is normally synthesized for a limited period of several hours⁴ during which the IFN- β mRNA accumulates and then rapidly disappears⁵. The shut-off occurs in the continuous presence of inducers and can be delayed by treatment with inhibitors of protein synthesis⁶. This treatment then allows the accumulation of IFN- β mRNA (superinduction)^{5,7}. It has been suggested that continuous protein synthesis supplies the cell with IFN- β mRNA degrading protein(s), thus involving the participation of other genes in IFN- β regulation⁶. We have analysed the induction of human IFN- β and interferon-associated genes in human fibroblast and lymphoblastoid cells^{8,9} and have previously reported the isolation and physical characterization of a 36-kilobase (Kb) region of the human genome including and surrounding an intact IFN- β gene¹⁰. The discovery that other poly(rI)poly(rC)-inducible genes are located in this cloned region presented the possibility that regulatory functions were acting through a common pathway on this family of genes.

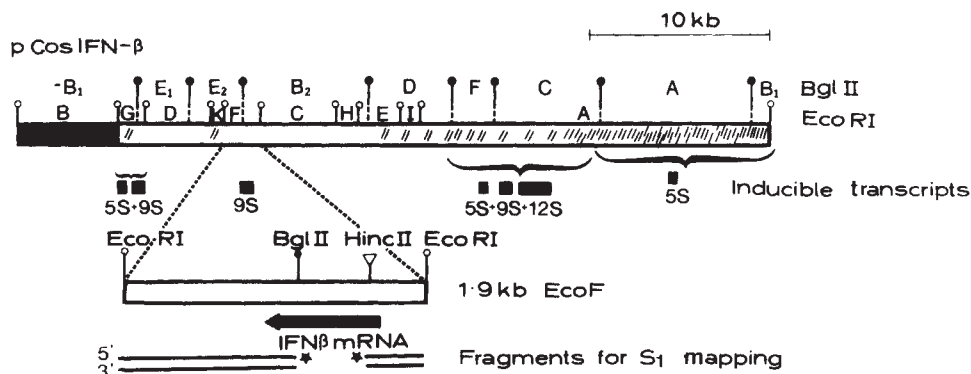
We describe here the production of human interferon in mouse L-cell clones containing defined fragments from the

human genome surrounding and including the human IFN- β (HuIFN- β) gene. The human IFN- β gene is clearly inducible with a spectrum of induction protocols which reflect the responsiveness of the mouse cells' own interferon genes rather than those of human fibroblasts. Because a 1.9-kb DNA fragment carrying only the structural gene of HuIFN- β is sufficient to allow induction, the neighbouring genes located on the human chromosomal DNA are not essential for the human interferon induction in mouse L cells.

Isolation of mouse L cells containing HuIFN- β cosmid DNA

We have co-transferred¹¹ cosmid pCosIFN- β DNA containing the human β -interferon gene¹⁰ (Fig. 1) together with plasmid pHCT9 2cos/tk DNA containing the herpes simplex virus thymidine kinase gene¹² into mouse Ltk⁻ cells. More than 20 hypoxanthine-aminopterin-thymidine (HAT)-resistant clones were isolated. The single clones were maintained in culture for over 50 generations of HAT medium before further studies at the molecular level were carried out. During this period the properties of individual clones with respect to interferon production remained unaltered. High molecular weight DNA was prepared from these cell clones and compared with DNA from human diploid fibroblasts (FS4 cells), mouse Ltk DNA and cosmid clone pCosIFN- β DNA by Southern blotting¹³ after restriction enzyme digestion. Figure 2 shows the result for two

Fig. 1 Physical map of pCosIFN- β and transcription units inducible in FS4 cells using poly(rI)poly(rC) inducer. The upper bar shows a linearized form of pCosIFN- β including the vector pJB8 (filled bar)¹⁰. The human DNA part is shown as an open box containing the hatched regions which indicate sequences repeated on human chromosomal DNA. Transcripts hybridizing to pCosIFN- β sequences are depicted as black bars. The lower enlargement of the 1.9-kb *Eco* F fragment shows the location of the IFN- β gene transcript and the fragments (*Eco*RI-BglII and *Hinc*II-EcoRI) which were used for S₁-mapping of the interferon transcript (see Fig. 4).



cell clones. Hybridization with the 1.9-kb *Eco* fragment of the cosmid clone gives rise to a single band of the original size in FS4 DNA and DNA from both cell clones. Due to middle and highly repetitive DNA contained in the cosmid clone¹⁰, hybridization of total cosmid to FS4 DNA leads to a background which obscures discrete bands.

Both mouse cell clones contain all *Eco* fragments present in the cosmid pCos IFN- β , indicating that most of the transferred DNA is in the original configuration. This could be explained by insertion of the transferred DNA into the genome in the form of concatenates. Such a tandem array would be a good substrate for cosmid rescue in which the *in vitro* packaging system is used to re-isolate the cosmid DNA directly out of the genome (according to ref. 11). When this experiment was carried out, pCosIFN- β clones could be re-isolated with an efficiency of ~20 clones per μ g of mouse hybrid 2₄ genomic DNA (G.G., unpublished results), thus supporting the hypothesis that some, if not all, of the cosmid copies are integrated in tandem. By comparing the band intensities after different exposure times, we estimated that clone 12₃ has taken up >20 and clone 2₄ >50 copies of the total cosmid.

Active human interferon can be induced in mouse cell clones

All cell clones tested showed low levels of constitutive human interferon production (10–100 U ml⁻¹). On 4 h induction with poly(rI)poly(rC) plus DEAE-dextran (25 and 100 μ g ml⁻¹, respectively), the interferon production in 19 out of 20 clones tested was enhanced to levels 4–1,000-fold higher than the constitutive base level production (Table 1). The levels of interferon production were determined by the antiviral assay with vesicular stomatitis virus as challenging virus on either FS4 or Vero cells (for assaying the human-specific activity) or mouse L cells (for assaying the mouse-specific activity)¹. Although mouse L cells are known to produce α - and β -interferons on induction¹⁴, tests on induced Ltk⁺ control cultures for cross-reactivity showed no interferon activity in either human FS4 or monkey Vero cells. Furthermore, the antiviral activity of purified HuIFN- β was 1,000-fold higher on human than mouse test cells.

In clone 2₄, the Newcastle disease virus (NDV)-induced levels of HuIFN- β exceeded levels obtained with superinduced human FS4 fibroblasts, whereas mouse IFN production in the clone containing the HuIFN- β gene remained unaffected by the presence of the heterologous DNA. The human IFN- β gene responded to induction in the same way as the mouse genes: in conditions of superinduction, a very low level of interferon was detected and induction with NDV was more efficient than with other inducers (Table 1a). These data on the dominance of the mouse cell over the response pattern of the human interferon gene agree with the findings of Graves and Meager¹⁵, who examined human interferon induction in human-mouse heterokaryotypes (from somatic cell fusions).

Wiranowski-Stewart *et al.*¹⁶ have shown that the cell death during superinduction in human fibroblasts is largely due to the actinomycin D treatment and that if another RNA inhibitor is

used, interferon induction may be repeated with the same cells at weekly intervals. We attempted repeated cycles of interferon induction with the mouse cells clone 2₄ using a 72-h cycle, and allowing 48 h for recovery in fresh medium. At least five cycles could be carried out without loss of interferon synthetic capacity (Table 1b).

Although there was little doubt that the interferon induced in the mouse cell clones and assayed on human cell lines was indeed HuIFN- β , we tested the characteristics of the protein further by immunological methods using monoclonal anti-HuIFN- β antibody¹⁷. The test involved gel electrophoresis of extracellular protein and electrophoretic blotting of this gel on

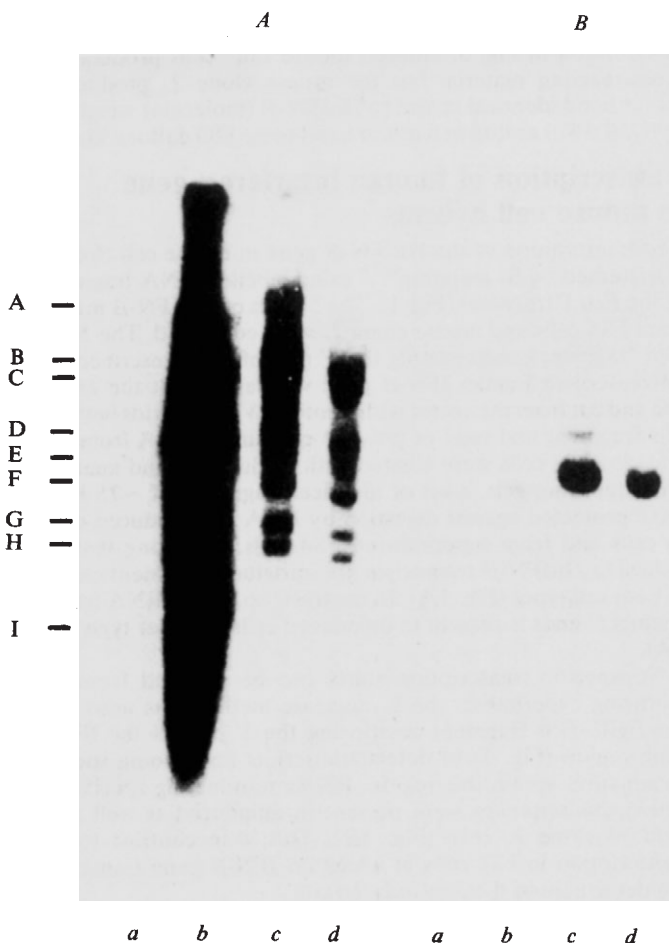


Fig. 2 Analysis of pCosIFN- β DNA in mouse L-cell clones. Mouse Ltk⁻ cells were transformed using the calcium phosphate technique^{17,32} with 250 μ g supercoiled pHC79-2cos/tk⁺DNA, 10 μ g supercoiled pCosIFN- β DNA and 10 μ g Ltk⁻ carrier DNA per 5×10^5 cells. Two established Ltk⁺ clones (12₃ and 2₄) were analysed as follows. High molecular weight DNA from the clones was cleaved with *Eco*RI. 12 μ g of DNA was run in each lane of a 1% agarose gel, transferred (blotted) to nitrocellulose paper¹³ and hybridized with either ³²P-labelled pCosIFN- β DNA (A) or *Eco* F fragment (B). a, Mouse Ltk⁻; b, FS4 human fibroblast DNA; c, Ltk⁺ clone 2₄; d, Ltk⁺ clone 12₃. A–I indicate the size of *Eco* fragments typical of pCosIFN- β as in Fig. 1.

Table 1 Production of specific interferons in human and mouse cells

<i>a</i> Cell type	Interferon titre (U ml ⁻¹)				Interferon type
	Without inducer	+poly(rI)poly(rC)	Superinduction	+NDV	
Human FS4 fibroblasts	<5 <1	1,600 <1	12,000 <1	2,000 <1	Human Mouse
Mouse Ltk	<5 <1	<5 130	<5 32	<5 300	Human Mouse
Mouse Ltk ⁺ +pCosIFN- β clone 2 ₄	50 <1	12,000 130	6,000 32	50,000 300	Human Mouse
Mouse Ltk ⁺ +pCosIFN- β clone 12 ₃	10 <1	3,200 130	ND	ND	Human Mouse
<i>b</i>					
	Day 1	Day 4	Human interferon titre (U ml ⁻¹)		
			Day 7	Day 10	Day 13
Clone 2 ₄	6,500	3,200	12,000	6,500	6,500
Clone 12 ₃	3,200	1,600	3,200	1,600	ND

a, The supernatants were screened on FS4 or Vero cells, specifically for human IFN, and on mouse Ltk⁺ cells, specifically for mouse IFN, in an antiviral test¹. Human IFN activity is given as international reference units and mouse IFN in arbitrary laboratory units. Induction with DEAE-dextran plus poly(rI)poly(rC) (100 and 25 $\mu\text{g ml}^{-1}$, respectively) was performed for 4 h in serum-free Dulbecco's minimal essential medium (DMEM). For superinduction the cells were incubated for 4 h in serum-free medium with 25 $\mu\text{g ml}^{-1}$ poly(rI)poly(rC) plus 50 $\mu\text{g ml}^{-1}$ cycloheximide and 3.5–4 h after induction with 5 $\mu\text{g ml}^{-1}$ actinomycin D. Induction with NDV Beaudette C was carried out by addition of 10 plaque-forming units of UV-inactivated NDV per cell in serum-free DMEM for 1 h. Following these induction periods the cells were washed thoroughly and incubated for a further 12 h in fresh medium plus 10% calf serum (10 ml per 2×10^7 cells) before assaying the IFN content in the medium. *b*, Confluent monolayers of L-cell clones 2₄ and 12₃ were induced for 4 h with DEAE-dextran-bound poly(rI)poly(rC) (see *a*). IFN produced after 12 h was in a minimal amount of serum-free medium (10 ml per 2×10^7 cells) after washing. The cells were allowed to recover for 48 h in medium plus serum and were then reinduced using the above protocol. ND, not determined.

to nitrocellulose paper ('Western blot'). Proteins having immunological cross-reaction with HuIFN- β were stained. As can be seen in Fig. 3, induced mouse Ltk⁺ cells produced no cross-reacting material but the mouse clone 2₄ produced a major band identical in size to HuIFN- β (molecular weight 19,500 (19.5 K)) as well as a minor band some 800 daltons smaller.

Transcription of human interferon gene in mouse cell hybrids

The transcription of the HuIFN- β gene in mouse cell clone 2₄ was studied by S₁ mapping^{18,19} using labelled DNA fragments of the Eco F fragment (Fig. 1). The 5' ends of the IFN- β mRNA from FS4 cells and mouse clone 2₄ were compared. The Hinc-Eco fragment encompassing the 5' part of the transcribed and untranscribed human IFN- β gene was labelled at the HincII site and cut from the vector with EcoRI⁹. When hybrids between this fragment and total or poly(A)-containing RNA from FS4 and clone 2₄ cells were treated with S₁ nuclease and analysed on sequencing gels, a set of identical fragments of ~75 bases were protected against digestion by RNA from induced clone 2₄ cells and from superinduced FS4 cells, indicating that the inducible HuIFN- β transcripts are initiated at an identical site in both cell types (Fig. 4A). In contrast, no IFN- β RNA having distinct 5' ends is present in uninduced cells of either type (Fig. 4A).

Nonspecific transcription starts can be deduced from the following experiment: the S₁ nuclease method was used with the BglII-Eco fragment comprising the 3' part of the IFN- β gene region (Fig. 1) to detect transcripts undergoing specific termination within this region. RNAs terminating specifically within this sequence were present in uninduced as well as in induced clone 2₄ cells (Fig. 4B). This is in contrast to the transcription in FS4 cells in which no IFN- β gene transcripts are detectable in the non-induced cell.

To clarify the nature of these transcripts, we examined interferon-specific RNA by Northern blotting²⁰ in clone 2₄, identifying interferon-specific transcripts by hybridization with nick-translated Eco F DNA (Fig. 5). A 9S RNA species corresponding to IFN- β mRNA predominated over the background only after induction. The background present in RNA from induced and non-induced cells reflects transcripts of heterogeneous length mostly longer than 9S. The transcription of this heterogeneous RNA in mouse clone 2₄ is probably due to multiple non-specific transcription starts far upstream of the specific start site which is characterized by a 'TATA box' and

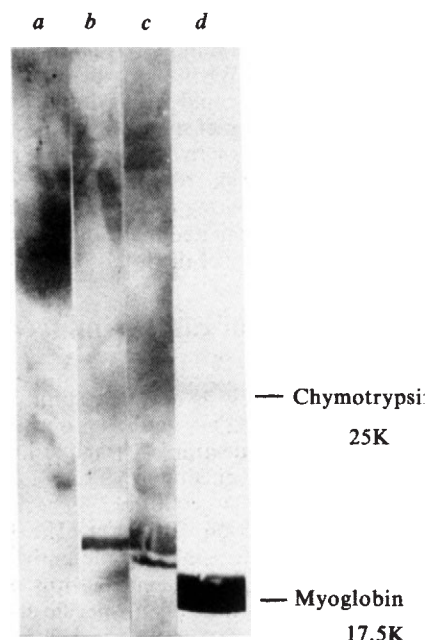
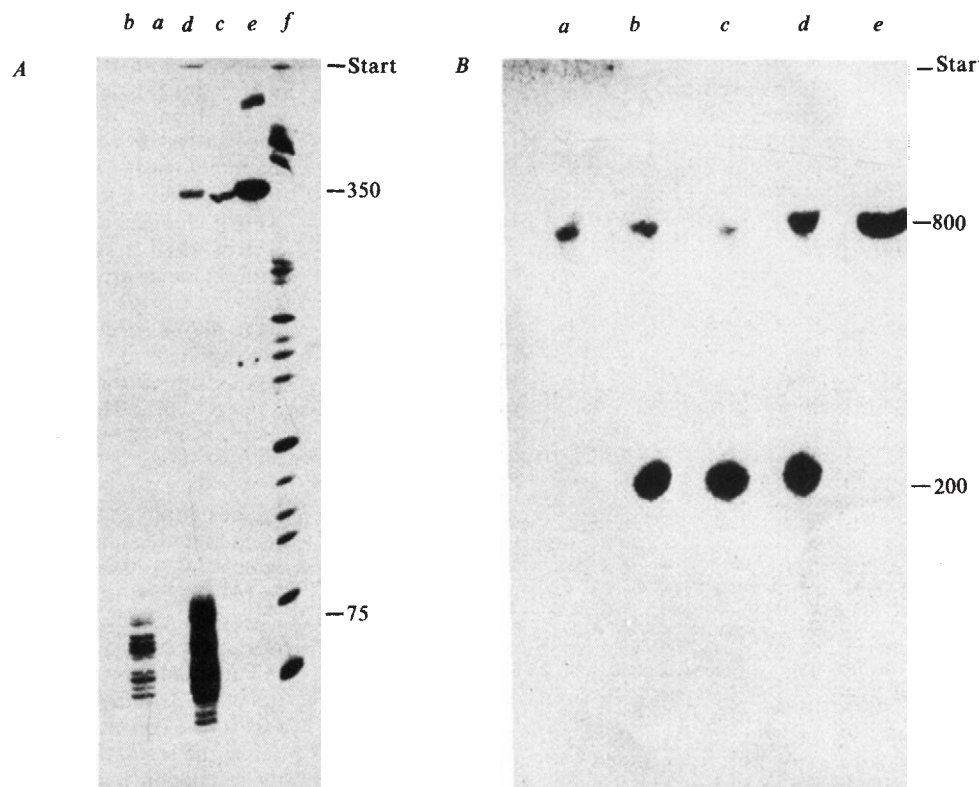


Fig. 3 Analysis of human IFN- β from a mouse cell hybrid using a monoclonal antibody-driven enzyme-linked immunosorbent assay. Supernatants were collected from mouse Ltk⁺ cells (*a*) or mouse Ltk⁺ clone 2₄ cells (*c*) at 12 h after a 4-h induction period (poly(I)poly(C) (25 $\mu\text{g ml}^{-1}$) + 100 $\mu\text{g ml}^{-1}$ DEAE-dextran in serum-free DMEM). These supernatants were precipitated with 10% trichloroacetic acid and the pellets washed three times in acetone (0 °C) and once with cold ether. After lyophilization the pellets were suspended in SDS-sample buffer. As a positive control, human IFN- β from FS4 human fibroblasts was used in position *b*. Molecular weight markers were run in lane *d*. All these samples were run on a 15% SDS-polyacrylamide gel according to Laemmli³³. This gel was blotted electrophoretically (a 'Western blot') onto a nitrocellulose sheet (Millipore) as described by Towbin *et al.*³⁴. The section of the blot containing the molecular weight markers (*d*) was stained with amido black. The other sections were tested for human IFN- β content by the following treatment: (1) 2 h at 25 °C in 5% bovine serum albumin (BSA)/5% horse serum to saturate nonspecific protein binding sites; (2) wash in Tris-buffered saline, pH 7.4; (3) mouse monoclonal (ascites fluid: IgM) anti-human IFN- β was added at a 1:100 dilution in 5% BSA/5% horse serum/0.01% sodium azide and incubated for 24 h at 4 °C. After a further washing step (4) the blot was soaked for 16 h in rabbit (IgG) anti-mouse IgM (1:500 in 5% BSA/5% horse serum) and after a further wash (5) was incubated for 16 h with peroxidase-conjugated goat (IgG) anti-rabbit IgG (1:500 in 5% BSA/5% horse serum). The blot was washed again and the colour reaction performed with *o*-diazimidine as substrate. Lane *b* contained 30,000 U of human IFN- β and *c* contained 40,000 U of human interferon (as measured on human FS4 cells.)

Fig. 4 S_1 nuclease mapping of HuIFN- β mRNA made in human and mouse L-cell clones. Total RNA was extracted from FS4 cells 4 h after superinduction and from mouse clone 2₄ cells which had been induced for 7 h with DEAE-dextran-bound poly(rI)poly(rC). The 32 P-end-labelled probes (compare Fig. 1) for mapping the 5' and 3' ends from huIFN- β mRNA were prepared as described elsewhere⁹. 1 μ g of poly(A)-containing RNA from FS4 cells and 25 μ g total RNA from mouse cells were used for hybridization with an excess of the end-labelled DNA fragment in 80% formamide buffer. Hybridization was performed at 42°C for 12 h. The 5' hybrids (A) were treated with a high concentration of S_1 -nuclease (5,000 U ml⁻¹) to reduce the amount of unhybridized DNA fragment. For 3'-end mapping, 1,000 U ml⁻¹ S_1 nuclease was used. The hybrids were denatured and run on a denaturing 6% acrylamide gel (A for the 5' probe) or a 2% alkaline agarose gel (B for the 3' probe), and the gels were dried and autoradiographed at -70°C. a, Uninduced FS4 RNA; b, induced FS4 RNA; c, uninduced clone 2₄ RNA; d, induced clone 2₄ RNA; e, undigested DNA fragment used for hybridization; f, 32 P-end-labelled pBR322 DNA digested with *Hae*III; sizes are indicated in bases.



other sequences typical of eukaryotic promoters^{10,21}. The protection of the complete *Hinc*-*Eco* fragment with RNA from induced and non-induced clone 2₄ cells (Fig. 4A, lanes c, d) reflects nonspecific transcripts which start 5' upstream of the *Eco* site.

The presence of human IFN- β RNA with distinct 5' ends in the induced mouse clone 2₄ is consistent with the production of HuIFN- β activity in this clone. Superinduced FS4 cells and induced mouse L cell hybrids express similar levels of HuIFN- β activity (Table 1a).

Transcription of pCosIFN- β DNA in mouse cell clones

In FS4 cells, in addition to IFN- β mRNA, several poly(rI)poly(rC) inducible transcripts can be detected which hybridize to the human DNA carried by pCosIFN- β : a small RNA of ~5S hybridizes to the *Bgl*II A, C and F fragments, a 9S RNA hybridizes to *Eco* G and A fragments and a transcript of ~12-13S hybridizes to fragment *Eco* A (ref. 8).

To determine whether the same pattern of RNAs is transcribed from the pCosIFN- β DNA in the mouse cell clones, we isolated total and poly(A)-containing RNA from hybrid cell clones before and at several times after induction. Transcripts from the transferred cosmid DNA were identified by hybridization to Northern blots of nick-translated DNA from pCosIFN- β or isolated DNA fragments. Heterogeneous transcripts were found corresponding to most parts of the transferred pCosIFN- β DNA, including the vector DNA (data not shown). In addition, specific bands were detected in the RNA from clone 2₄ after induction with poly(rI)(rC) plus DEAE-dextran (Fig. 5). The presence of the background of heterogeneous transcripts in the uninduced state prevented us from clearly identifying the large transcripts corresponding to *Eco* fragments A, G and F. Transcripts similar to the RNAs from FS4 cells can be seen as faint bands above the background (data not shown). Nevertheless, a small inducible transcript of ~5S (Fig. 5) which hybridises specifically to the *Bgl*II A, C and F fragments, could be clearly identified (data with individual probes not shown). This RNA is most probably identical to that seen in induced FS4 cells.

Regulation of human IFN- β expression in mouse L cells

To examine the influence of flanking sequences on the regulation of interferon gene expression, we co-transferred pBR13 carrying the 1.9-kb *Eco* F fragment of pCosIFN- β in pBR325 together with pHC79-2cos/tk into mouse Ltk⁻ cells. Again, screening of single resistant cell clones for interferon production and induction showed a low constitutive base level production and variable inducibility of HuIFN- β (data not shown), indicating that the 1.9-kb *Eco* fragment is sufficient for interferon production and response to induction.

Comparison of the kinetics of human interferon induction and shut-off in cell clones containing either pCosIFN or pBR13 DNA showed no significant difference. This indicates that the neighbouring DNA regions present in pCosIFN- β are not necessary for the regulation of the human β -interferon gene in mouse L cells.

The biological assay provides a sensitive and specific tool for measuring even low amounts of interferon production. We have examined transient expression of pCosIFN- β and pBR13 DNA in mouse Ltk⁻ cells and, using the calcium phosphate technique, found production of up to 100 U ml⁻¹ of human interferon in the cell supernatant up to 3 days after transformation. However, there was no further induction by DEAE-bound poly(rI)poly(rC) during this period, although endogenous mouse interferon was inducible to a normal extent. This indicates that the ability of the IFN- β gene to respond to induction develops with the time after transfer.

Discussion

The availability of cloned genomic DNA and its introduction into heterologous mammalian cells allows the potential study of gene regulation in a foreign environment. Many attempts to find a gene which maintains its inducibility in a heterologous background have been unsuccessful (for example, chicken ovalbumin and rabbit β -globin genes in mouse cells^{22,23}). However, recent findings with cloned mouse mammary tumour virus (MMTV) and rat α_2 -microglobulin genes in mouse Ltk⁻ cells have shown that hormone responsiveness is maintained in the new environment²⁴⁻²⁶.

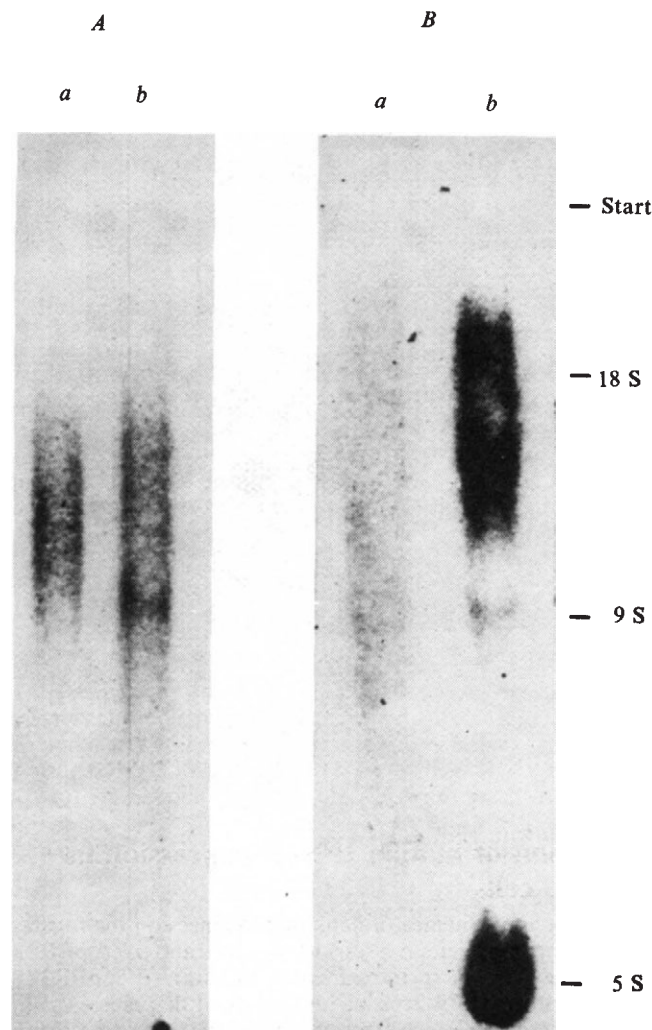


Fig. 5 RNA transcribed from pCosIFN- β in mouse Ltk⁺ clone 2₄. Total and poly(A)-containing RNA from clone 2₄ cells before and at different times after induction with DEAE-dextran-bound poly(rI)poly(rC) were run on 2% formaldehyde gels²⁰. The RNA was transferred to nitrocellulose filters and hybridized to nick-translated Eco F DNA (A) or pCosIFN- β (B)⁸. After washing, the dried filter was autoradiographed at -70 °C using an X-ray intensifying screen. Ethidium bromide-stained RNA served as size marker. A, 5 μ g of poly(A)-containing RNA from uninduced (a) and 7 h-induced (b) cells. B, 20 μ g of total RNA from uninduced (a) and from cells 12 h after induction (b). Nearly identical results with less intensive hybridization signals were obtained with RNA from clone 12₃ (not shown).

We have shown that in human fibroblasts and mouse L-cell clones containing the 36-kb piece of human DNA, the induced IFN- β mRNA is identical. However, a constitutive background of heterogeneous transcription starts is present in most mouse L-cell clones. A basal level of human interferon activity in the absence of inducer may be due to translation of IFN- β mRNA

with heterogeneous size or, more probably, to a very low level of specific transcription, as is observed with chicken ovalbumin and rabbit β -globin genes in mouse L cells^{22,23}.

The human IFN- β produced, as measured by a species-specific test based on biological activity, was further defined immunologically with monoclonal antibody. Although the major protein species detected in the medium had a molecular weight identical to normally glycosylated HuIFN- β (molecular weight 19K) a smaller band was also observed. How these protein bands are actually related to normal human interferon remains an open question. The identity in size of the major band allows speculation that most of the HuIFN- β induced is glycosylated to the same extent as normal human IFN- β . (Mouse interferons have a larger molecular weight—24K and 36K (ref. 14).) The presence of the second smaller band could imply either a proteolytic cleavage or heterogeneity in the glycosylation.

We have previously shown that some of the transcribed regions adjacent to the huIFN- β gene are differentially regulated as, for example, in a comparison of human fibroblasts and lymphoblastoid cells⁸. Although these other transcripts are not as well defined as the IFN- β mRNA itself, it was clear that at least one other 'gene' (for the 5S RNA) was specifically inducible in the mouse clones to an even more dramatic extent than in human fibroblasts. As these neighbouring regions have little or no effect on the inducible transcription of HuIFN- β in mouse L cells, we conclude that if these transcribed regions are involved in the regulation of IFN- β expression in human fibroblasts, their function has been replaced by mouse gene products in the clones.

The finding that transient expression of the huIFN- β gene is unaffected by the presence or absence of inducer is an interesting phenomenon. It has been shown that transient expression of several genes occurs in a large proportion of transformed cells^{27,28}, although very few eventually stably maintain and express the gene. During stabilization of such clones the transferred DNA which is organized in a high molecular weight form (transgenomic form) becomes associated with chromosomes²⁹. Most of the transiently expressed DNA is not in such a transgenomic organization³⁰. The inducibility of the HuIFN- β gene correlates with its establishment in a stably integrated form.

During preparation of this manuscript, Ohno and Taneguchi³¹ reported inducible transcription of RNA complementary to the Eco F fragment in mouse cells transformed with SV2 Eco gpt Eco F DNA.

We thank Dr Rentschler AG (now Bioferon AG) for providing us with induced FS4 cells and human β -interferon standards, Karin Littmann for technical assistance, Dr A. C. R. Samson for providing us with a range of NDV subtypes and Dr W. Lindenmaier for helpful discussion. A preliminary report of this work was presented at the 17th Harden Conference on Interferon, Ashford (September, 1981).

Note added in proof: Mantei and Weissmann³⁵ have demonstrated inducibility of human IFN- α 1 mRNA in NDV-induced mouse L cell clones.

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Sequence homology and structural comparison between the chromosomal human α_1 -antitrypsin and chicken ovalbumin genes

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The human chromosomal α_1 -antitrypsin gene has been cloned. This gene is approximately 5 kilobase pairs long and contains three intervening sequences in the peptide-coding region. DNA sequences coding for the amino and carboxyl termini of α_1 -antitrypsin have been identified. Human α_1 -antitrypsin and chicken ovalbumin show significant sequence homology and belong to a common protein super-family. Yet the number, position and size of intervening sequences reveal that the two genes are dissimilar.

α_1 -ANTITRYPSIN is an important plasma protease inhibitor which is capable of inhibiting a wide variety of serine proteases, including proteases involved in blood coagulation, fibrinolysis and kinin generation¹⁻⁴. Although α_1 -antitrypsin is a plasma protein of hepatic origin, its primary site of physiological action may not be in the blood. There is evidence that it is transported by passive diffusion into the alveolar structure of the lung and protects this organ from destruction by polymorphonuclear leukocyte elastase⁵⁻⁹. α_1 -Antitrypsin inhibits elastase by forming an equimolar complex of enzyme and inhibitor with an association rate constant of $6.5 \times 10^7 \text{ mol}^{-1}$ (refs 10, 11). This association rate constant is 3.4×10^5 and 1.3×10^6 times greater than that of human plasmin and thrombin respectively. The balance between elastase and α_1 -antitrypsin in the lung is perturbed genetically in individuals with an inborn deficiency of α_1 -antitrypsin. This imbalance can lead to hydrolysis of the elastic fibres of the lung by elastase, resulting in permanent damage to the alveolar structure¹²⁻¹⁴. Accordingly, individuals with α_1 -antitrypsin deficiency are 20-30 times more likely to develop chronic obstructive pulmonary emphysema than is the general population^{15,16}.

The synthesis of α_1 -antitrypsin is controlled by an autosomal and allelic system^{4,17}, and over 30 different phenotypes of the protein have been identified^{18,19}. In order to study the normal and abnormal α_1 -antitrypsin genes, we have screened a human genomic DNA library using a cDNA probe prepared from baboon liver^{20,21}. We report here the isolation and characterization of several clones containing the human chromosomal α_1 -antitrypsin gene.

Overlapping genomic α_1 -antitrypsin clones

Sixteen independent phage isolates were obtained when 2×10^6 plaques from the human genomic DNA library²² were screened using the baboon α_1 -antitrypsin cDNA clone²⁰ as hybridization probe. Subsequent analysis of the isolates indicated that they originated from four independent clones— α AT135, α AT35, α AT80 and α AT101. The four clones were analysed by restriction mapping and Southern hybridization using as probes a *Mbo*II fragment of pBa α 1a1 DNA, which contains the 3'-terminal region of the baboon cDNA²⁰, and a *Hha*I fragment of pBa α 1a2 DNA which is a baboon cDNA clone lacking only ~100 nucleotides at the 5' end of the mRNA²¹. These results have established the orientation of the human α_1 -antitrypsin gene and indicate that the entire gene may reside within a 9.6 kilobase (kb) *Eco*RI DNA fragment in the human genome (Fig. 1).

Mosaic structure of the human α_1 -antitrypsin gene

The overall structure of the human α_1 -antitrypsin gene was established by electron microscopy of hybrid molecules formed between the cloned chromosomal DNA and baboon α_1 -antitrypsin mRNA. The mature mRNA consists of ~1,400 nucleotides. DNA was denatured thermally and hybrids were formed subsequently in conditions favouring RNA-DNA hybridization but not DNA-DNA reassociation. Figure 2a shows two representatives of 15 such molecules and the corresponding line

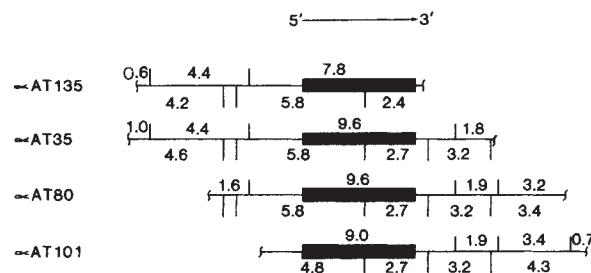
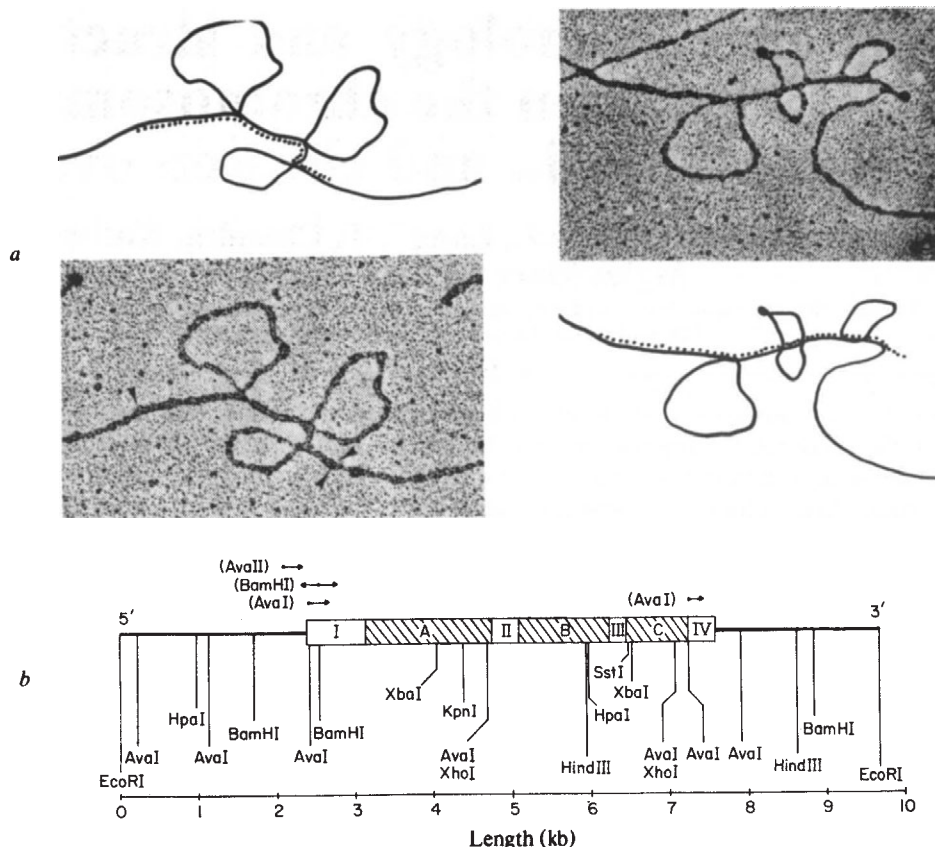


Fig. 1 Schematic representation of overlapping genomic clones of the human chromosomal α_1 -antitrypsin gene. ■, The α_1 -antitrypsin gene region; →, transcription orientation. The vertical lines above and below the horizontal lines represent *Eco*RI and *Hind*III cleavage sites respectively. The numbers indicate the distances between restriction sites in kilobases. The signs at the end of each clone represent the artificial *Eco*RI sites created during construction of the genomic DNA library by the method of Lawn *et al.*²². Approximately 2×10^6 recombinant Charon 4A phages were screened for clones containing α_1 -antitrypsin gene sequences by the *in situ* plaque hybridization technique of Benton and Davis⁵⁶, using an amplification procedure previously described⁵⁷. A hybridization probe for the α_1 -antitrypsin gene was prepared by *Pst*I and *Mbo*II digestion of a partial-length cDNA clone containing the 3'-terminal sequence of baboon α_1 -antitrypsin mRNA²⁰. The resulting 120 bp fragment was purified by preparative polyacrylamide gel electrophoresis⁵⁸ and the DNA fragments were labeled with ³²P by nick translation according to the procedure of Maniatis *et al.*⁵⁹. As the probe was only 120 bp long and of baboon origin, the library screening by cross-hybridization was carried out in slightly relaxed conditions ($6 \times \text{SSC}$ at 62°C). The α_1 -antitrypsin gene-containing recombinant phages were plaque-purified three times and phage DNA was prepared as previously reported⁶⁰. The genomic clones of the human α_1 -antitrypsin gene were digested with a variety of restriction enzymes in conditions suggested by the manufacturer, and the resulting DNA fragments were separated by agarose gel electrophoresis. DNA fragments were transferred from the agarose gel to nitrocellulose filters by the method of Southern⁶¹ and the filters were treated according to the procedure of Denhardt⁶². Hybridization was carried out overnight with the labelled baboon cDNA probes in $6 \times \text{SSC}$ at 68°C . Differential hybridizations involving either the 5' or the 3' portion of a baboon cDNA probe were performed as described above after bidirectional transfer of DNA fragments from agar gels onto nitrocellulose filters by the method of Smith and Summers⁶³.

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Fig. 2 Structure of the human chromosomal α_1 -antitrypsin gene. *a*, Electron micrographs and line drawings of hybrid molecules of the human chromosomal α_1 -antitrypsin gene and baboon liver poly(A)-RNA. —, Single-stranded genomic human DNA; ·····, baboon α_1 -antitrypsin mRNA. Hybrids of the cloned human chromosomal α_1 -antitrypsin gene ($10 \mu\text{g ml}^{-1}$) with the baboon liver poly(A)-RNA ($20 \mu\text{g ml}^{-1}$) were formed in 70% deionized formamide containing 100 mM Tris-HCl (pH 7.6), 10 mM Na^+ -EDTA and 30 mM NaCl. The mixture was heated at 80°C for 5 min and incubated at 55°C for 3 h. Immediately after incubation, samples were prepared for electron microscopy by diluting 0.1–0.5 μg of nucleic acid with 100 μl of a solution containing 70% formamide, 0.1 M Tris-HCl (pH 8.4), 0.01 M Na^+ -EDTA and 100 $\mu\text{g ml}^{-1}$ of cytochrome *c*. The mixture was spread onto a hypophase of distilled water. Samples were picked up on collodion-coated 300-mesh copper grids, stained with uranyl acetate, rotary-shadowed with platinum-palladium and examined at 80 kV using a JEOL 100 C electron microscope. Molecules were measured using a Numonics electronic planimeter with $\phi\text{X174 RD DNA}$ as the internal standard. *b*, Schematic representation of the human chromosomal α_1 -antitrypsin gene. Structural segments ($\square$) are indicated by Roman numerals and intervening sequences ($\blacksquare$) by capital letters. The transcriptional orientation of the α_1 -antitrypsin gene in human genomic DNA is from left to right. All restriction digests were performed in conditions suggested by the manufacturer. Horizontal arrows above the gene represent sequenced DNA regions (see Fig. 3). In each case, the arrow shows the direction of sequencing and the restriction enzyme site (in parentheses) that was labelled (closed circle).



drawings. It is evident that there are three intervening DNA loops (introns) of various sizes in the human α_1 -antitrypsin gene. The poly(A) tract in the mRNA is clearly visible in the hybrid molecule shown in the right-hand panels, thereby confirming the orientation of the gene. Indeed, when αAT135 DNA was cleaved with *EcoRI* before hybrid formation with the baboon mRNA, the smallest intervening DNA loop was very close to one end of the DNA molecule (not shown). Numonic measurements of the hybrid molecules have indicated that the exon regions I, II, III and IV are approximately 0.71, 0.33, 0.13 and 0.27 kb long respectively. Introns A, B and C are 1.45, 1.15 and 0.8 kb long respectively, and all of them appear to be located in the 3' half of the mRNA.

To characterize further the human chromosomal α_1 -antitrypsin gene, the 9.6 kb *EcoRI* DNA fragment was subcloned into an *EcoRI* site of pBR322. The resulting clone, pAT9.6, was analysed by restriction mapping and Southern hybridization. Four exon segments were identified in the 9.6 kb *EcoRI* DNA fragment using a combination of enzymes that do not cut the baboon α_1 -antitrypsin cDNA insert in pBa α 1a2 (ref. 21). These results (Fig. 2*b*) confirmed the existence of three introns in the human α_1 -antitrypsin gene. As the baboon cDNA clone was used to map the human chromosomal gene, the restriction enzymes that did not cleave the baboon cDNA might actually have cleaved the exons in the human gene due to sequence divergence between the two species. This possibility seemed unlikely, however, because at least two hexanucleotide recognition sites are present in each of the three introns in the human chromosomal DNA and absent from the baboon cDNA sequence. The presence of only three introns in the peptide-coding region of the human chromosomal α_1 -antitrypsin gene has recently been confirmed by DNA sequence analysis (G.L.L., unpublished results).

The 5'- and 3'-terminal sequences of the human α_1 -antitrypsin gene

Southern hybridization analysis between different portions of the baboon cDNA clone and human genomic fragments generated by digestion of pAT9.6 revealed DNA fragments which hybridize uniquely with either the 5' or the 3' end of the cDNA probe. By DNA sequencing, we identified fragments of the human genomic DNA that code for amino acids at both the amino- and carboxyl-terminal regions of human α_1 -antitrypsin (Fig. 3). The two regions are ~ 5 kb apart, which is in good agreement with our estimates of the size of the gene, based on the electron micrographs. The amino acid sequence at the amino-terminal region is consistent for 30 of the 33 residues with that previously published for human α_1 -antitrypsin²³. Different amino acids include Lys 10, His 20 and Ile 26, which were reported as Glu, Ser and Leu respectively²³. The DNA sequence corresponding to the amino-terminal region of the protein was confirmed by sequencing both strands of the genomic DNA. Furthermore, the residues in question are identical to those determined for baboon α_1 -antitrypsin²¹. The amino acid sequence containing 32 residues at the carboxyl end of the mature protein was also deduced from the genomic DNA sequence. This amino acid sequence is in complete agreement with that previously published for α_1 -antitrypsin²⁴. Also, the genomic DNA sequence was identical with the corresponding nucleotide sequence of a human α_1 -antitrypsin cDNA clone²¹.

The first ATG start codon at the 5' end of the α_1 -antitrypsin gene is located 24 amino acids upstream from the amino-terminal Glu residue in the mature protein (Fig. 3*a*). This region seems to code for a typical signal peptide, which is removed from the mature protein during intracellular processing, before extracellular transport. This signal peptide resembles other

signal peptides in having an amino-terminal methionine residue, a hydrophobic core flanked by regions of more polar residues, a small uncharged amino acid at the putative cleavage site, proline at position -5 and a length of ~15–30 amino acids²⁵. Furthermore, there appears to be a 'TATA box'²⁶ at position -25 to -31 of the gene (Fig. 3a), which resembles the consensus sequence, TATA_{TAT}_{ATA}, proposed by Cordon *et al.*²⁷. The transcription start point in eukaryotes also has a consensus sequence, PyCAPyPyPyPyPy (A = position +1; Py = pyrimidine), based on 20 genes²⁷. This is less obvious in the α_1 -antitrypsin gene but can be tentatively assigned to bases -2 to 6 (Fig. 3a). Benoist *et al.*²⁸ have pointed out an additional consensus sequence (GG_TCAATCT) further upstream (~80 residues) from the transcription start point which may also be important in the control of gene expression. This 'CAAT' box is less recognizable in the α_1 -antitrypsin gene.

The hexanucleotide AAUAAA occurs in eukaryotic mRNAs approximately 20 bases upstream from the point of poly(A) attachment²⁹. However, in the α_1 -antitrypsin gene, a sequence of AUUAAA is present in this position (Fig. 3b). This somewhat atypical sequence has also been reported for mouse pancreatic α -amylase³⁰, human leukocyte interferon³¹ and chicken lysozyme³² mRNAs.

Sequence homology between human α_1 -antitrypsin and chicken ovalbumin

An unexpectedly high degree of amino acid sequence homology between the carboxyl-terminal 152 residues of human α_1 -antitrypsin and those of chicken ovalbumin was originally observed by Hunt and Dayoff³³. Based on the assumption that the rates of change for both proteins lie between 9.8 and 18 PAMs (point mutations per 100 residues) per 100 million years, which are accepted values for animal lysozyme and for pancreatic secretory trypsin inhibitor³⁴, it was proposed that the divergence time between the two proteins could be over 500 Myr ago. They then proposed that the two proteins belong to a common super-family and that they diverged before or during early vertebrate evolution³³. We have subsequently determined the complete amino acid sequence of baboon α_1 -antitrypsin and shown that the two seemingly unrelated proteins have a 24% amino acid sequence homology²¹. The amino acid sequences of the two proteins were also examined by a matrix-plot computer analysis^{35,36}, which reveals not only the extent of sequence homology, but also the positions of homologies, and relatedness of two sequences is displayed as a diagonal line on the plot. The diagonal line apparent in such a plot between α_1 -antitrypsin and ovalbumin has confirmed that homology between the two proteins exists at corresponding positions throughout the molecules (data not shown).

Structural comparison of human α_1 -antitrypsin and chicken ovalbumin genes

Gilbert³⁷ has proposed that intervening sequences may serve to separate portions of the coding region of genes corresponding to the structural-functional domains for their corresponding proteins. The gene structures for chicken ovomucoid³⁸, the β -chain of haemoglobin³⁹ and the heavy chain of immunoglobulin⁴⁰ support this hypothesis. As human α_1 -antitrypsin and chicken ovalbumin apparently belong to the same super-family, it was predicted that the two genes would contain similar exon-intron patterns³³. The number and locations of the introns in the human α_1 -antitrypsin gene as determined by electron microscopic and restriction mapping were thus compared with those in the chicken ovalbumin gene (Fig. 4). The chicken ovalbumin gene contains seven introns and their exact locations in the gene have been established by DNA sequencing^{41–43}. The human α_1 -antitrypsin gene contains only three introns, which are located in the 3' half of the corresponding mRNA

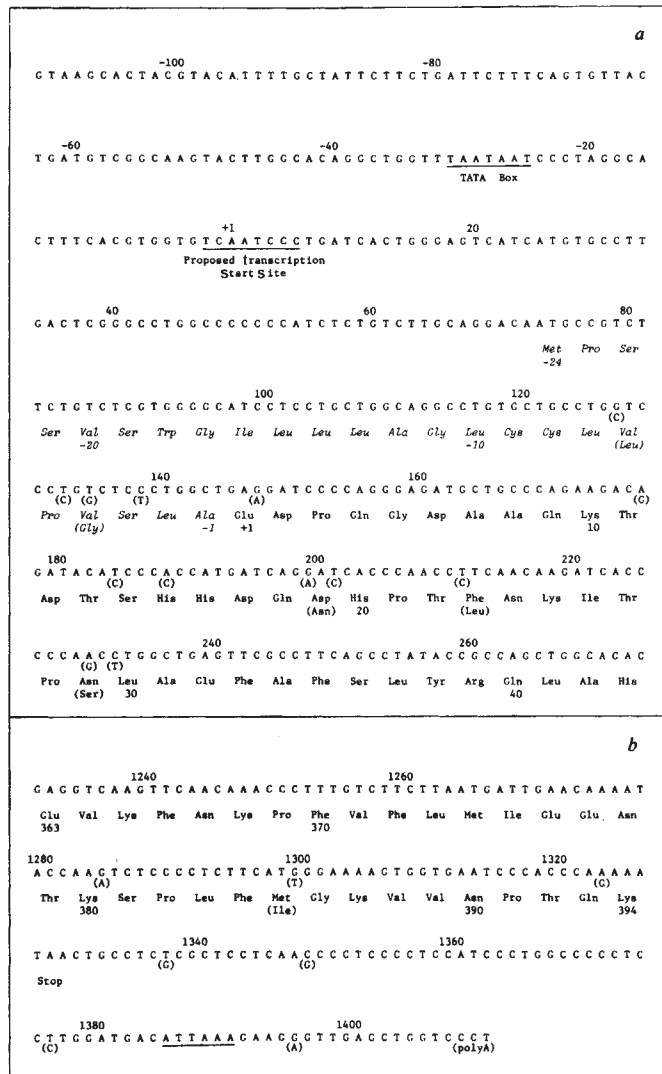


Fig. 3 Nucleotide sequences and the deduced amino acid sequences at the 5' (a) and 3' (b) termini of the human α_1 -antitrypsin gene. Also included are the corresponding bases or amino acids (shown in parentheses) observed for baboon cDNA²¹. Numbering for the nucleic acids is based on the proposed transcription start point (+1), that of the amino acids on the amino terminal residue of the mature protein (+1). Proposed transcription recognition sites are underlined. The treatment of the DNA fragments with bacterial alkaline phosphatase, labelling of the 5' termini with [γ -³²P]ATP using T4 polynucleotide kinase, isolation of labelled DNA fragments by preparative polyacrylamide gel electrophoresis and chemical degradation of end-labelled DNA fragments were performed according to the procedure of Maxam and Gilbert⁵⁸. Numbering for the nucleic acids at the 3' end and the corresponding amino acids is based on the transcription start point shown in a and homology with a baboon cDNA clone²¹. The underlined sequence corresponds to a site related to mRNA polyadenylation described by Proudfoot and Brownlee²⁹.

(Fig. 2), whereas all seven introns in the chicken ovalbumin gene are located in the 5' half of the mRNA. Based on the electron microscopic map, the only introns that might share a common site in the two genes are intron A in the human α_1 -antitrypsin gene and intron E or F in the chicken ovalbumin gene (Fig. 4). Intron A in the human α_1 -antitrypsin gene has recently been located by DNA sequencing at a position 27 and 21 amino acids away from ovalbumin introns E and F respectively (Fig. 5). In the region shown, 21 of 60 amino acids (35%) and 86 of 180 nucleotides (48%) are identical. However, a comparison of nucleotide sequences for the 5' and 3' ends of the introns with the corresponding coding regions of the other gene shows only 28% (α_1 -antitrypsin intron A) and 25% (ovalbumin intron F) identity of bases, values close to the 25% level

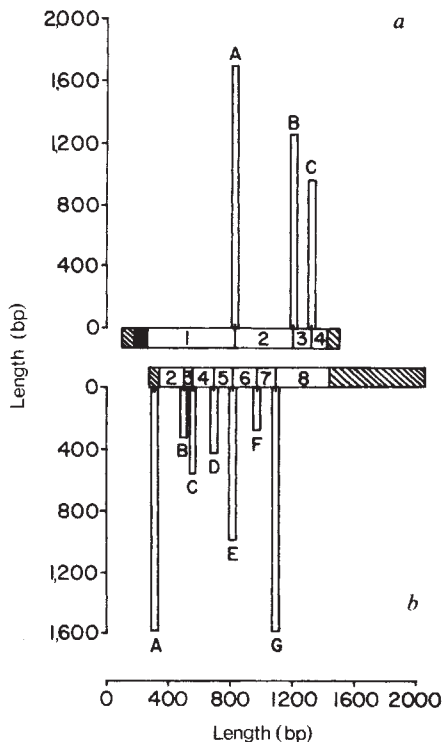


Fig. 4 Comparison of the genes for α_1 -antitrypsin (a) and ovalbumin (b). □, Coding regions for the two proteins; ■, position of a proposed 'signal' sequence in α_1 -antitrypsin; ▨, flanking noncoding regions of the corresponding mRNA transcripts. Alignment of the two genes was based on amino acid sequence homology for the two proteins²¹. Intervening sequences are shown by the vertical bars. The size and position of the coding and intervening sequences for ovalbumin have been published elsewhere⁴¹⁻⁴³.

expected from random DNA sequences. These data provide clear evidence for the difference between the two genes in terms of position, size and composition of introns in a region of high protein sequence homology.

Evolutionary origin of the human α_1 -antitrypsin and chicken ovalbumin genes

At present it is impossible to determine conclusively whether human α_1 -antitrypsin and chicken ovalbumin arose by conver-

gent or divergent evolution. The lack of similarity in structure between the two genes is certainly not unexpected if convergent evolution were the pathway. We have previously shown that overall amino acid sequence homology between a third member of this protein super-family, human antithrombin III³³, which is another plasma protease inhibitor, and α_1 -antitrypsin and ovalbumin is 28% and 31% respectively²¹. The cloning and characterization of the human chromosomal antithrombin III gene will permit the comparison of the mosaic structures of these genes, which may elucidate the evolution of the gene family.

It would be very intriguing if these genes did arise by divergent evolution. In contrast to the other multi-gene families such as the globins⁴⁴ and vitellogenins⁴⁵, where the number and positions of introns are rigidly conserved, the α_1 -antitrypsin and ovalbumin genes contain different numbers of introns which are located at completely different sites in the exonic sequences. This observation suggests that the ancestral gene to α_1 -antitrypsin and ovalbumin may have been duplicated before the addition or deletion of intervening DNA sequences. The fact that the X-Y-ovalbumin gene family^{46,47} in chicken has retained the number and positioning of the introns suggests subsequent gene duplication events involving the ancestral ovalbumin gene.

The differences in structure of the human α_1 -antitrypsin and chicken ovalbumin genes resemble those in the actin gene family. The actin genes of yeast^{48,49}, dictyostelium⁵⁰, drosophila⁵¹ and sea urchin⁵² have one, zero, one and two introns respectively; yet although the exons have retained extensive sequence homology and resemble those of the mammalian cytoplasmic actins, the positions of the introns are completely distinct. It has thus been suggested that the current models for the functional and evolutionary conservation of locations of introns may not be valid for all genes⁵³, and that some introns are vestiges of transposon-like elements that have been inserted into genes, become fixed and subsequently diverged in nucleotide sequence⁵¹. It has also been pointed out, however, that owing to the limited numbers of introns present in the actin gene family, these genes could also have evolved by random deletion of multiple introns present in the primordial actin gene⁵¹. Precise deletion of an entire intron has been reported for the rat insulin gene, resulting in the existence of two genotypic alleles⁵⁴. Similar observations have been made for the mouse α -globin gene⁵⁵. In the case of α_1 -antitrypsin and ovalbumin however, if indeed the ancestral gene to these proteins contained 10 or more introns and the two genes arose by random intron deletion, a number of introns would be expected to share common sites in the exons of the two genes.

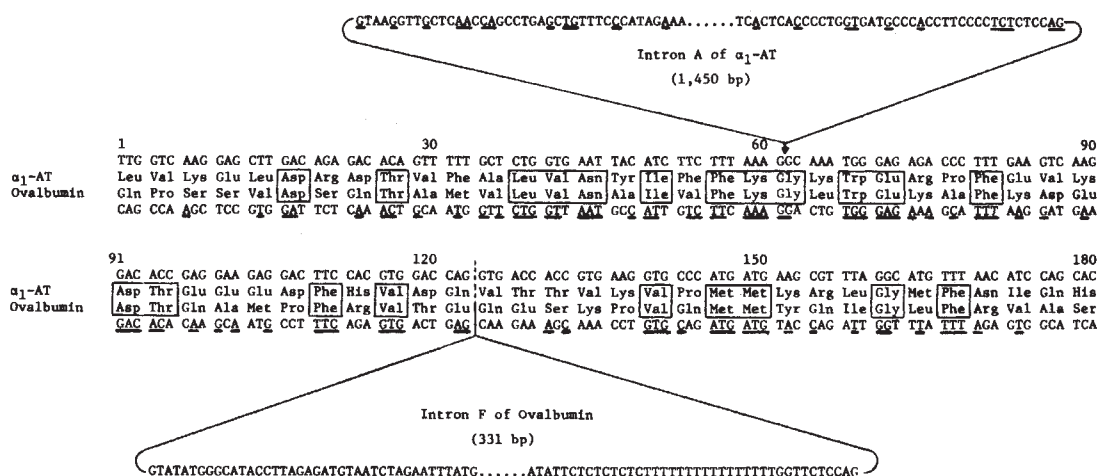


Fig. 5 Comparison of amino acid and nucleic acid sequences of splice junctions for α_1 -antitrypsin (α_1 -AT) intron A and ovalbumin intron F. Nucleic acid sequences corresponding to the mRNA molecules are shown in the 5' → 3' direction. Identical bases for the two genes in the coding regions are underlined in the ovalbumin sequence. Forty bases at each end of each intron are shown for comparison with corresponding continuing base sequences of the other gene. Underlined bases in the introns are identical to those in the coding region of the opposite gene. The position of each intron is shown by a vertical line joining the ends of each loop. The first amino acids shown are residues 172 and 162 of mature α_1 -antitrypsin and ovalbumin respectively. Ovalbumin intron E is located 81 base 5' to α_1 -antitrypsin intron A (data not shown).

The fact that the positions of the introns in the two genes show no overlap makes this hypothesis less attractive. Consequently, at least some of the introns could be the vestiges of transposable elements that had been inserted into pre-existing exons of the two genes after their divergence from an ancestral gene.

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Molecular characterization of two myosin heavy chain genes expressed in the adult heart

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Four cDNA clones containing cardiac myosin heavy chain (MHC) inserts have been characterized. Hybridization and nucleotide sequence analysis identify two different MHC genes coding for proteins of different length that are both specifically expressed in the adult heart. The nucleotide and amino acid sequences reveal a highly conserved structure in the light meromyosin portion of MHC from striated muscle tissues.

A GROWING body of evidence suggests that several muscle-specific contractile proteins are encoded by multi-gene families. In invertebrates, birds and mammals, different actin genes^{1–3} and different myosin light chains (MLCs)^{4–6} are expressed in muscle and non-muscle tissues. Recent observations obtained by peptide mapping^{6–8}, immunological cross-reactivity^{8,9} and DNA analysis^{10–12} have now documented at the protein and gene levels that at least 10 myosin heavy chain (MHC) genes are expressed in the rat. In other vertebrates the MHC genes are also encoded by a multigene family^{12,13}. Each muscle type (skeletal, cardiac and smooth) has tissue-specific MHCs that differ from the non-muscle (cytoplasmic) MHC^{12–17}. However,

the MHCs in different striated muscle tissues show a considerable degree of sequence homology not only within the same species but also across the evolutionary scale¹². Despite these similarities, peptide and enzymatic analysis has shown that cardiac myosin is different from the myosins in other striated muscles^{5,6}. The existence of distinct cardiac myosins in atrial and ventricular cells has been demonstrated in rat and chicken hearts by immunohistochemical and kinetic approaches^{15,18}. Like skeletal muscle MHC^{6–8}, cardiac muscle MHCs seems to be developmentally regulated^{6,15,19}. In several mammals, three myosin ventricular isozymes with different Ca²⁺-ATPase activity and different MLCs and MHC composition, have been identified^{6,15,19}. These three isozymes have been named V₁, V₂ and V₃ on the basis of their increasing electrophoretic mobility¹⁵. In the rat, a double transition between these isozymic forms is correlated with the age of the animal^{15,19}. V₁, with the

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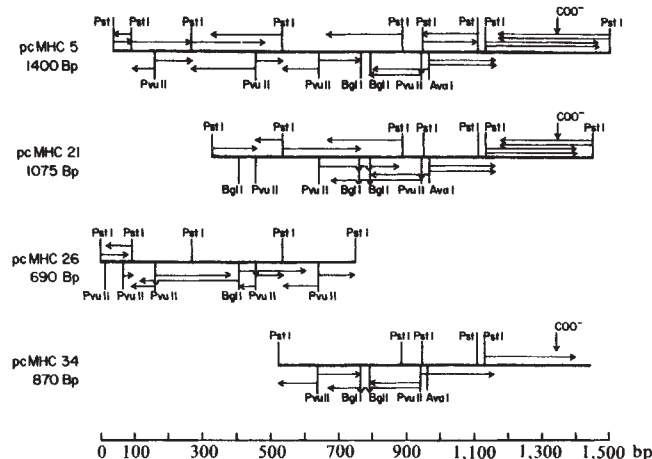


Fig. 1 Restriction endonuclease maps of cDNA clones containing MHC inserts. The recombinant cDNA clones were prepared as described elsewhere²⁴. Single and double digestion with endonucleases *Pst*I, *Pvu*II, *Ava*I and *Bgl*I were in 25- μ l reactions containing 6 mM Tris-HCl pH 7.5, 6 mM MgCl₂, 6 mM 2-mercaptoethanol and appropriate salt recommended by the various suppliers, for 2 h at 37°C. The relative position of the enzyme restriction sites was determined by the method of Smith and Birnstiel⁴². Solid bar represent the MHC cDNA inserts. Orientation from left to right corresponds to the 5'-3' orientation of the mRNA strand. The nucleotide length covered by the inserts is indicated. Sequencing strategy for the cMHC cDNAs clones: Horizontal arrows indicate the direction and length of each fragment sequenced. cDNA clones (20 μ g) digested with the appropriate enzymes (indicated at the starting point of each arrow) were labelled at the 5' termini with [γ -³²P]ATP (2,000 Ci mmol⁻¹) or at the 3' termini with 3'-[α -³²P]dATP (cordycepin 5' triphosphate 1,000-3,000 Ci mmol⁻¹) as described elsewhere⁴³. Single-end labelled fragments were obtained either by digestion with a second restriction endonuclease or by strand separation, followed by polyacrylamide gel electrophoresis. The labelled fragments were localized on the gel by X-ray autoradiography, excised from the gel and electroeluted. The DNA sequencing was performed according to the method of Maxam and Gilbert⁴².

highest Ca²⁺-ATPase activity, appears at birth and is prevalent throughout life; V₃ and the heterodimer V₂ are the only fetal forms and reappear in the second month of postnatal life. In the adult, the isozymic distribution can be modulated in different physiological and pathological conditions²⁰⁻²³. The differences seen in the polypeptide composition of the MLCs and MHCs of embryonic and adult hearts suggest that different myosin genes are expressed at specific developmental stages of the rat^{6,21}.

To improve our understanding of the molecular mechanisms responsible for the developmental and tissue-specific expression of the cardiac MHC genes and the possible physiological and pathological implications of the isozymic transitions, we have constructed recombinant cDNA clones that contain cardiac MHC mRNA sequences. We show here that at least two different but closely related cardiac MHC genes are expressed in the adult rat. These two genes are apparently developmentally regulated. The structure and nucleotide sequence of these cardiac MHC cDNA clones are discussed and compared with those of skeletal muscle MHCs.

Isolation of MHC cDNA clones from cardiac muscle

The extent of cross-homology within the MHC gene family¹² makes it difficult to identify the MHC gene(s) expressed in different cell types and at a particular time of development. Therefore, identification of gene-specific sequences becomes essential when studying MHC gene regulation at the molecular level. To this end, we constructed a cDNA library by the G-C tailing method in *Pst*I site of pBR322 using standard cloning techniques²⁴, with poly(A)⁺ RNA isolated from the ventricles of a 3-month-old rat (Wistar strain) as template for cDNA synthesis. We identified 16 recombinant plasmids containing MHC inserts, by the Grunstein and Hogness colony hybridization procedure²⁵, using as probe for MHC sequences the

320-base pair (bp) internal *Pst*I restriction fragment of the embryonic skeletal muscle MHC cDNA clone pMHC₂₅ (ref. 24).

It has been previously shown that pMHC₂₅, obtained from L₆E₉ myotubes^{26,27}, contains sequences that are highly conserved within the sarcomeric MHC gene family and cross-hybridizes to cardiac mRNA^{10,12}. However, this procedure limited the detection to those cardiac MHC cDNA clones that cross-hybridize to the embryonic skeletal MHC and contain the region corresponding to the insert used as probe. Therefore, the 16 cDNA clones represent a minimum estimate of the MHC cDNA clones in the cDNA library.

The four cardiac MHC cDNA clones that contain the longest inserts (690-1400 nucleotides) were selected for further analysis. Note that because MHC mRNA is 7,100 nucleotides long²⁴, the longest clone, pCMHC₅, contains 23% of the MHC mRNA sequence. As shown in Fig. 1, pCMHC₅, pCMHC₂₁, pCMHC₂₆ and pCMHC₃₄ have very similar restriction endonuclease maps and have been aligned to facilitate comparison. A remarkable feature of these maps is the frequency of sites for the restriction endonucleases *Pst*I and *Pvu*II that recognize the nucleotide sequences CTGCAG (Leu-Gln) and CAGCTG (Gln-Leu), respectively. This feature seems to be a characteristic of the vertebrate MHC genes so far studied^{13,24,28,29}. The *Pst*I and *Ava*I sites are conserved in the region where the different clones overlap. Taking pCMHC₅ as a reference, pCMHC₂₁ has an additional *Bgl*I site at coordinate 400 of the diagram. pCMHC₂₆ also has a *Bgl*I site at position 400 and an additional *Pvu*II site at position 80. pCMHC₃₄ covers a region of 900 nucleotides that shows identical restriction enzyme sites in all four cDNA clones. On the basis of these observations, the MHC cDNA clones represented in the cardiac cDNA library cover approximately the same portion of MHC mRNA and can be divided into two classes, one represented by pCMHC₂₁ and pCMHC₂₆ and the other by pCMHC₅; pCMHC₃₄ could belong to either class. Each of these two sets probably originates from a single mRNA species, thus suggesting a minimum of two cardiac MHC genes expressed in the ventricular myocardium of the heart used for the cDNA cloning.

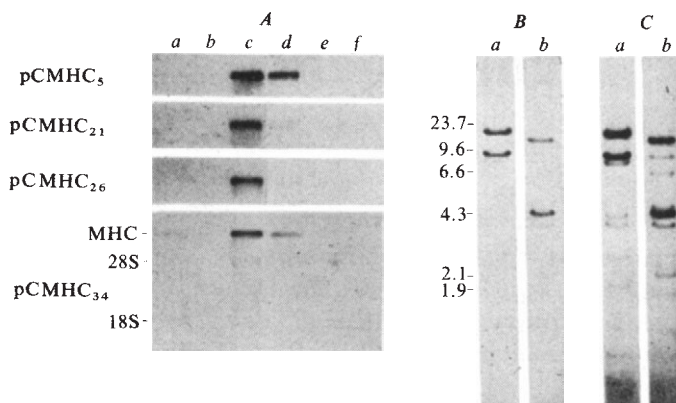


Fig. 2 Identification of MHC cDNA sequences in RNA from different rat tissues and in rat genomic DNA. Total cytoplasmic RNAs were isolated from different tissues by the hot phenol procedure⁴⁴ and from L₆E₉ cells by using guanidine hydrochloride^{45,46}. RNA (5 μ g per lane) was fractionated on 35% formaldehyde, 1% agarose gels and blotted onto nitrocellulose⁴⁷. DNA was isolated from rat liver nuclei⁴⁸, and 10 μ g were digested with restriction endonucleases in 50 μ l reaction buffer (see Fig. 1 legend), separated on 1% agarose-TAE buffer (40 mM Tris-acetate pH 7.8, 5 mM Na-acetate, 1 mM EDTA) and transferred onto nitrocellulose³⁰. cDNA probes were radiolabelled by nick-translation in the presence of [α -³²P]CTP and [α -³²P]TTP (400 Ci mmol⁻¹)³¹. 10⁶ c.p.m. were used per gel lane for hybridization. Hybridization washes were in 0.1 $\times$ SSC, 0, 1% SDS at 65°C. A, hybridization of pCMHC₅, pCMHC₂₁, pCMHC₂₆, pCMHC₃₄ to rat RNA from: a, L₆E₉ myotubes; b, L₆E₉ myoblasts; c, adult cardiac muscle; d, adult skeletal muscle; e, uterine smooth muscle; f, NRK fibroblasts. Marker indicates migration of 28S and 18S ribosomal RNAs. B, hybridization of pCMHC₅ to Wistar rat DNA digested with: a, *Eco*RI; b, *Hind*III. Size markers indicated in kilobases are from *Hind*III-digested λ phage DNA. Exposure of the autoradiogram was for 3 days. C, same as in B except that exposure of the autoradiogram was for 14 days.

Fig. 3 Nucleotide sequence of cardiac MHC cDNA clone inserts. The Maxam and Gilbert³² method was used for sequence determination. The sequencing strategy is depicted in Fig. 1. pCMHC₂₆ (nucleotides 1–790) pCMHC₂₁ (nucleotides 350–1,340) and pCMHC₃₄ (nucleotides 500–1,340) sequences that represent portions of the same cardiac MHC gene product have been joined into a continuous 1,340-nucleotide sequence. In pCMHC₃₄, the longer nucleotide sequence of the untranslated 3' end (nucleotides 1,341–1,351) reads ATTTTGCTGTG(A)90. The nucleotide and the derived amino acid sequences are written 5' to 3' and NH₂ to COOH, respectively, in the upper two lines. The nucleotide and amino acid sequences of pCMHC₅ that represent the other cardiac MHC gene product (nucleotides 40–1,384) are in the bottom two lines. In pCMHC₅, nucleotides and amino acid are written only where different from that of pCMHC_{26,21} and ₃₄. The dashed line indicates identical sequence to that of pCMHC_{21,26,34}.

A previous study on the genomic organization of the MHC sequences has determined that a minimum of eight genes encoding striated muscle MHCs are detected in the rat genome¹⁰. We attempted to determine which and how many striated MHC genes could be identified as cardiac specific. The four cardiac cDNA clones were ³²P-labelled³⁰ and hybridized individually to restriction digests of rat DNA (Wistar strain) prepared by the Southern blotting technique³¹. pCMHC₅ (Fig. 2, panel *B*) and pCMHC_{21,26} and ₃₄ (not shown) generate the same pattern when hybridized to genomic DNA. At high stringency, this pattern consists mainly of two strongly hybridizing fragments

of approximately 8 and 21 kilobases (kb) in the *Eco*RI digest (lane a) and 4.5 and 18 kb in the *Hind*III digest (lane b). The other hybridization bands that become more apparent on long exposure of the autoradiograms (Fig. 2, panel C) reflect cross-hybridization to segments corresponding to other skeletal muscle genes¹⁰. However, due to the poor resolution of the gel system within this size range it is not possible to ascertain whether each band represents only one or more gene fragments.

Considering that the cardiac MHC cDNA clones have neither *Eco*RI nor *Hind*III restriction sites, it is probable that each pair of strongly hybridizing fragments represents indeed two different cardiac MHC genes. Alternatively, the hybridization pattern could be produced by a single MHC gene, interrupted by an intervening sequence containing the appropriate restriction sites. This possibility is very unlikely because internal *Pst*I restriction fragments of pCMHC₅ and pCMHC₂₁ generate the same hybridization pattern as the entire cDNA clones (data not shown).

This result suggests that more than one cardiac MHC gene is represented in the genome. However, a clear determination of how many and which cardiac genes encode for the MHC mRNAs represented by cDNA clones is hampered by the obvious sequence homology within these genes.

Nucleotide sequence analysis of the cardiac MHC cDNA clones

To obtain unequivocal information on the number of cardiac MHC genes represented in the MHC cDNA clones, understand the extent of their homologies and identify gene-specific sequences, we determined the complete nucleotide sequence of the four cDNA clones by the method of Maxam and Gilbert³².

The sequence analysis shows that the cDNA clones comprise the sequence of the 430 carboxyl terminal amino acids, the complete untranslated 3' end and a portion of the poly(A) track of MHC mRNA. These four clones represent the transcription products of two distinct MHC genes that have 95% homology in the coding region but are clearly different in their untranslated 3' ends. The sequences of pCMHC₂₁, pCMHC₂₆ and pCMHC₃₄ overlap over 400 base pairs and clearly represent transcription products of the same MHC gene. However, in pCMHC₃₄, the untranslated 3' end is 11 nucleotides longer than that of pCMHC₂₁. This extra sequence precedes the poly(A) track of the MHC mRNA (see Fig. 3 legend), thus indicating heterogeneity in the polyadenylation site. The sequence of pCMHC₅ corresponds to a segment of a different MHC gene product.

The continuous nucleotide and derived amino acid sequences of these two MHCs, one represented by pCMHC₂₁, pCMHC₂₆ and pCMHC₃₄ and the other by pCMHC₅, are shown in Fig. 3. A characteristic of this portion of the two cardiac MHC mRNAs

is the high G + C content (33.7% G and 26.9% C) and the low U content (11.2%). This feature, together with the presence of several inverted repeats, allows the formation of highly stable hairpin structures ($\Delta G = -12$ to -17.5 kilocalories). A computer search did not reveal the presence of significant direct repeats in either sequence.

Comparison of the two cardiac MHC mRNA sequences shows very close similarities in this portion of the molecules. Long stretches of almost complete identity are interrupted by single nucleotide changes that occur at 25 to 100 base intervals. A long segment of conserved sequence, with only two base substitutions, extend from nucleotides 663 to 1,050. However, the two sequences are strikingly different in two regions: from nucleotide 360 to 433, where there is a 30% base mismatch, and from nucleotide 1,260 to the end of the molecules where the sequences completely diverge. This 3' end divergence has several interesting features. In pCMHC_{21/26} this segment, which codes for a longer MHC protein with two extra amino acids, includes the last 21 nucleotides of the coding sequence, the termination codon UAA and a short untranslated 3' end of 60 nucleotides. Moreover, in this mRNA, the poly(A) track starts 10 nucleotides after the AATAAA sequence, thought to be the poly(A) addition signal³³. In pCMHC₅, the divergent 3' coding portion is only 15 nucleotides long. Furthermore, the termination codon UAG precedes an untranslated sequence of 100 nucleotides. In this mRNA the poly(A) track starts 18 nucleotides after the AATAAA sequence. The drastic differences in nucleotide sequence and in length of the 3' ends of the two molecules confirm unambiguously that at least two cardiac MHC genes are expressed in the adult rat.

Amino acid sequence derived from the cardiac MHC cDNA clones

The coding sequence of pCMHC_{21/26} and pCMHC₅ represent the 430 carboxyl-terminal amino acids of two MHCs. This portion of the molecule forms an α -superhelical coiled-coil dimeric structure³⁴ known as the light meromyosin (LMM). The LMM amino acid sequence of these two cardiac MHCs have 97% homology. Moreover, there are striking similarities between these sequences and rat²⁹ and rabbit skeletal MHCs³⁴. The amino acid composition derived from the two cardiac sequences is characteristic of LMM³⁴. Half of the sequence is constituted by five amino acids (Glu, Gln, Leu, Lys, Ala). As expected, there are no Pro residues that disrupt the α -helix. The codon utilization frequency is strongly biased for G or C in the third position. For instance, Glu is coded 89% of the time by GAG; Gln, 98% CAG; Leu, 65% CTG; Lys, 90% AAG and ALA, 62% GCC. It merits mention that the codon usage for the body wall MHC in *Caenorhabditis elegans* is different from that of the rat cardiac MHCs, even though these two proteins show substantial homology (J. Karn, personal communication).

Fifty per cent of the nucleotide substitutions between the two cardiac MHCs occur at the third position and do not change the corresponding amino acid (Fig. 3). The few amino acid changes observed are conservative in hydrophobicity and/or charge. This is so even in the two portions of the molecule where most amino acid substitutions are clustered: residues 120–150 in pCMHC_{21/26} and the carboxyl-terminal ends of the two cardiac MHCs. Although the carboxyl-terminal sequences differ in length and diverge in amino acid composition, both terminate with two glutamic acid residues (Gln-Lys-Ile-His-Asp-Glu-Glu in pCMHC₂₁ and Gly-Leu-Asn-Glu-Glu in pCMHC₅). The carboxyl-terminal Glu-Glu is a common feature for all MHC sequences so far obtained^{29,34}.

From a structural point of view, the cardiac LMM sequences present a characteristic periodicity in the distribution of non-polar groups and charged residues. Non-polar, hydrophobic residues follow in general a 3,4,3,4 spacing, conforming to a stabilized α -helical structure as described for the tropomyosin molecule³⁵ and other MHCs³⁴. Occasional deviation of this

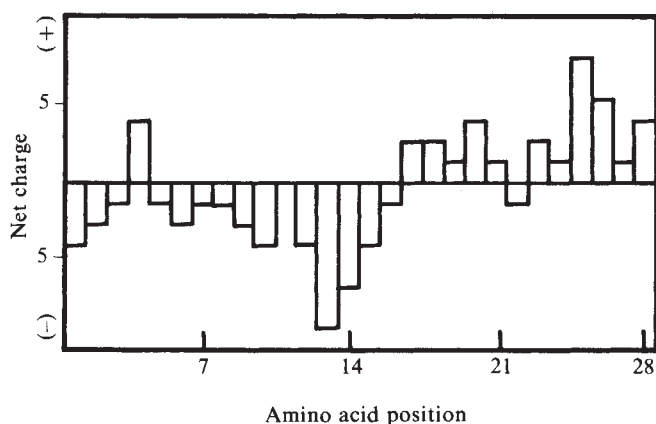


Fig. 4 Distribution of charged residues in the LMM portion of the cardiac MHC 'encoded' by pCMHC_{21/26}. The LMM was divided into periods of 28 residues. The relative position of each amino acid within the period is represented on the x axis. Positive and negative net charge at each position is recorded on the y axis.

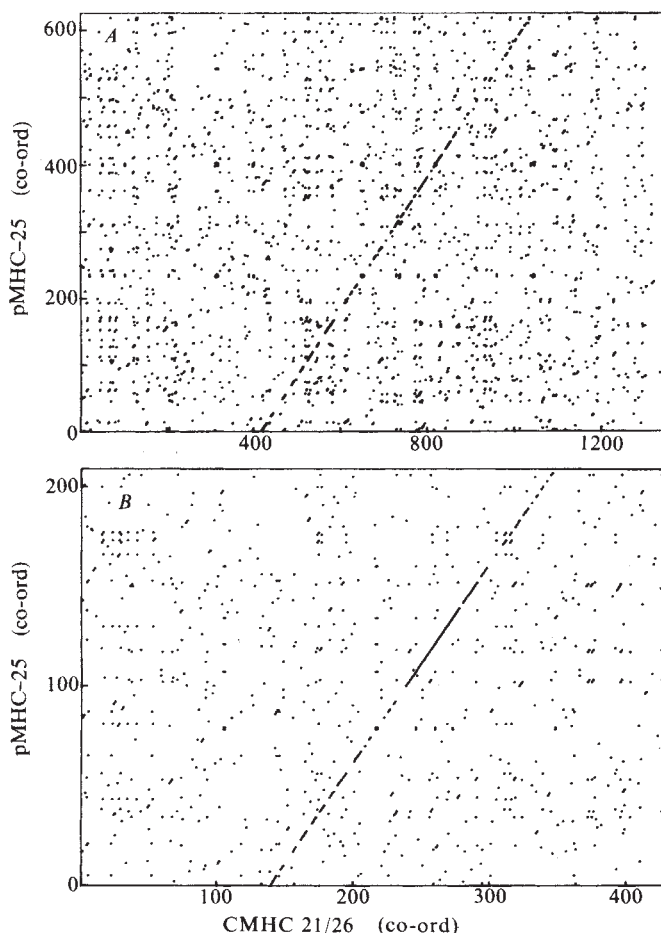


Fig. 5 Computer graphic representation of sequence homology in pCMHC_{21/26} and pMHC₂₅. A computer program was designed to detect and represent graphically homology between two sequences displayed on the x and y axis, respectively (R. Medford, unpublished). The computer compares sequentially a given number of nucleotides (or amino acids) from the sequence displayed on the x axis to each position in the sequence on the y axis. A homology match will generate a dot at the corresponding coordinate. Continuous homology is represented by a diagonal segment covering the length of the entire corresponding sequence. Displaced sequence identities, outside the main homology axis, are represented by short diagonal segments at their relative positions. **A**, comparison of the nucleotide sequence of pCMHC_{21/26} and pMHC₂₅. pMHC₂₅ covers nucleotides 415–1,045 of the sequence of pCMHC_{21/26}. Homology of five nucleotides in a row is required for a dot placement. This setting permits 50% third-base substitutions to be considered as homology match. **B**, comparison of the amino acid sequence of pCMHC_{21/26} and pMHC₂₅. An homology match is two amino acids in a row.

pattern, where the spacing is 3,3,4,4, is also observed. Clusters of positively and negatively charged residues are well separated and consist in most cases of sequential repeats of the same amino acid, for instance, Lys-Lys-Lys (positive charge) and Glu-Glu-X-Glu (negative charge). Charged clusters of identical sign show an optimal periodicity of 28 residues (see Fig. 4).

These structural features, which are conserved in rat^{28,29} and rabbit³⁴ skeletal muscle MHCs, as well as in body wall MHC of *C. elegans* (J. Karn and A. D. McLachlan, personal communication), are probably relevant to the formation and maintenance of the coiled-coil secondary structure of the LMM and to the organization of the myosin molecules in the thick filament. Interaction between charged groups within adjacent myosin chains might confer stabilizing properties for the assembly of the thick filament. Computer modelling experiments on the MHC in *C. elegans* have shown (J. Karn and A. D. McLachlan, personal communication) that surface charges, clustered on the outer surface of the α -helix, are best neutralized when two myosin filaments, aligned in parallel, are shifted by 98 residues. This would produce a 146 Å axial displacement of the myosin molecules. The displacement agrees very closely with the X-ray

diffraction data showing myosin cross-bridges in the thick filament, arranged in a helical configuration with a 143 Å periodicity³⁶.

Cardiac and skeletal muscle LMMs have highly homologous sequences

Sequence conservation in MHC genes from striated muscle is recognizable not only within the same species but also among species as distant as nematode, sea urchin, rat and human¹². To explore the distribution and the extent of sequence conservation between two MHCs from different striated muscles, we compared the sequences of cardiac MHC cDNA (pCMHC_{21/26}) and embryonic skeletal MHC cDNA (pMHC₂₅)²⁸, using a computer program designed to analyse and represent graphically sequence homologies (R. Medford, unpublished). The computer graphic display of such analysis is shown in Fig. 5A. The two sequences show close similarities as represented by the homology axis. Highly conserved domains, illustrated by continuous segments, are interspersed with regions of non-homology, represented by interruptions in the diagonal line.

One half of the restriction fragment of pMHC₂₅, which was used as a probe to screen the cardiac cDNA clones and other MHC cDNA and genomic clones (nucleotides 300–450 in pMHC₂₅), is 92% homologous to the cardiac clones and to other MHC cDNA clones from skeletal muscle²⁹.

The computer analysis of the amino acid sequence comparison of the two proteins 'encoded' by pMHC₂₅ and pCMHC_{21/26} is presented in Fig. 5B. Comparison of Fig. 5A and B clearly shows that amino acid sequence homology between these two cDNA clones is higher than the nucleotide sequence homology (compare nucleotides 300–450 with amino acids 100–150 on the pMHC₂₅ axis). Moreover, distribution of charged and non-polar residues is conserved in the segment of protein encoded by pMHC₂₅ (ref. 28). Structural and functional requirements of the sarcomeric MHC might account for such a feature.

The cardiac MHC genes are developmentally regulated

Age-dependent changes of cardiac myosin isozymes have been observed in several mammals^{15,16,18}. In the 12-week-old rat, cardiac myosin is 80% V₁ while the fetal forms V₃, and the heterodimer V₂, constitute the remaining 20% of the myosin¹⁸. As the cardiac MHC cDNA clones were constructed from cardiac mRNA of a 3-month-old animal, one set of cDNA

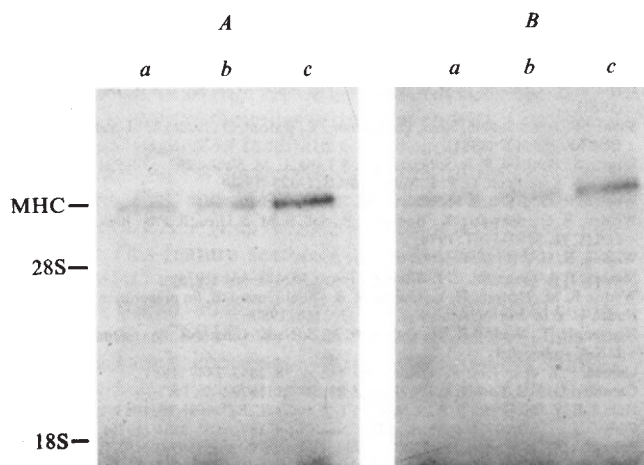


Fig. 6 Identification of cardiac MHC cDNA clones in rat cardiac RNA from different stages of development. Hybridization of pCMHC₅ (**A**) and pCMHC₂₁ (**B**) to rat cardiac RNA from: **a**, 20-day-old fetuses; **b**, 1-day newborn; **c**, 3-month adult. Samples were prepared as described in Fig. 2 legend. Hybridization washes were in 0.1×SSC, 0.1% SDS at 55 °C.

clones could represent the MHC in the major adult cardiac myosin V₁ and the other set could represent the MHC in the fetal cardiac myosin V₃. To investigate this possibility, the cDNA clones were hybridized to equal amounts of cardiac RNA isolated from rats at different stages of development, when either V₁ or V₃ myosin isozyme prevails^{15,18}. The result of such an experiment (Fig. 6) shows that both pCMHC₅ and pCMHC₂₁ (each containing the 3' end of the respective mRNA sequence) hybridize preferentially to MHC mRNA from the adult heart (panels A and B, lane c) and hybridize weakly to fetal and newborn RNA (panels A and B, lanes a and b). In fact, both entire clones, as well as their respective untranslated 3' ends (data not shown), show an increase in intensity of hybridization from the 20-day-old fetus and 1-day-old animal (lanes a, b) to adult MHC mRNA (lane c), which closely agrees with the reported data on the ontogeny of the V₁ isozyme^{15,18}. However, neither pCMHC₅ nor pCMHC₂₁ hybridizes preferentially to the 20-day-old fetal cardiac mRNA where 70% of the myosin is presumably the embryonic type V₃. Based on the assumption that the steady-state level of MHC mRNA, as a relative fraction of the total RNA population, does not change dramatically during heart development, these observations strongly suggest that in the rat not one, but at least two different adult-specific MHC genes are expressed in the ventricular myocardium. The weak hybridization to fetal MHC mRNA could reflect expression of these two cardiac MHCs early in development.

This conclusion is not necessarily in contradiction with previous reports on the existence of only one adult specific cardiac MHC isozyme^{15,18}. Based on the amino acid sequence, the two adult cardiac MHCs described here would be undistinguishable, at least in the LMM portion, by any of the techniques used so far to characterize the cardiac MHC at the protein level. Whether the high degree of sequence homology between these two MHC extends throughout the entire length of the molecule remains to be determined.

Discussion

The results reported here clearly show that a minimum of two adult-specific cardiac MHC genes are expressed in the ventricles. The existence of these two MHC genes raises several questions. What is their biological and physiological significance? Are these two genes expressed, in a coordinated fashion, in the same cell? At what precise stage of the heart development and at what level are they being transcribed? Are they expressed in other muscle tissues? Some of these questions

can now be approached by using the gene-specific sequences at the untranslated 3' termini of the mRNAs.

Class switches from fetal to adult gene(s) have been described for globin³⁷, skeletal muscle MHC⁸ and other systems³⁸. The isozymic transition of cardiac MHC during development requires modulation of the expression of at least two sets of MHC genes (fetal and adult). The remarkable feature of the cardiac MHC genes is that, in the rat, as well as in several other mammals¹⁸, the level of expression of fetal and adult MHC genes varies throughout the lifespan of the animal. Moreover, the expression of these genes can also be modulated in a reversible fashion by physiological conditions such as mechanical overload and level of circulating hormones^{15,21-23,39-41}. The adaptive regulation of expression of the MHC genes in cardiac tissue, as well as the existence of several MHC genes in other striated muscles^{8,10-13} offers an excellent model in which to study the control mechanisms that determine quantitative, temporal and spatial expression of specific members of the MHC gene family.

There is an apparent paradox in the multiplicity of the MHC genes and their intra- and interspecies sequence homology. The nucleotide sequence of the adult cardiac, embryonic and adult skeletal MHC mRNAs^{28,29} suggests that the sarcomeric MHC genes have evolved by duplication of a common ancestor gene. Furthermore, the amino acid sequence homology and the conservation of charged and non-polar residues indicate that secondary and possibly tertiary structure is very similar in the LMM portion of the different rat^{28,29} and rabbit³⁴ MHCs. These features indicate that the MHC genes are under evolutionary pressure for the conservation of certain sequences that might be relevant to the structure of the sarcomere. However, the large number of MHC genes points toward precise structural and/or kinetic requirements of each muscle, at various stages of development, that are best fulfilled by the corresponding isozyme. To understand the biological meaning of the MHC multi-gene family and its significance to muscle physiology, the structural and biochemical properties, as well as the subcellular location of each MHC isozyme, need to be determined.

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LETTERS TO NATURE

 **γ -ray sources as
comptonized X-ray sources****E. E. Fenimore, R. W. Klebesadel, J. G. Laros
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γ -ray burst spectra have often been fit by optically thin thermal bremsstrahlung. However, at the high temperatures implied by such fits ($kT \sim 300$ keV), the free-free cross-section is so much smaller than the Compton cross-section that Compton scattering might dominate the spectral formation processes. We have investigated the possibility that emission mechanisms based on Compton scattering can also fit the data. In particular, Monte Carlo calculations have been used to compare the γ -ray burst spectral data with black-body spectra which have undergone inverse comptonization by a much hotter, overlying plasma. The best-fit parameters suggest that the underlying black body is an X-ray source ($kT_{\text{BB}} \sim 2.4$ keV) while the overlying, comptonizing plasma has $kT_e \sim 150$ keV and a column density of 4×10^{24} electrons cm^{-2} . Such a model would also naturally explain some of the unusual γ -ray burst spectra. In particular, a low energy (~ 400 keV) cutoff in the spectrum of GB781119 can be explained as a Wien peak produced by a higher column density in the comptonizing region, and two-component spectra (for example, GB790329) can be explained by a lower column density.

Recently, several γ -ray burst spectra have been measured with sufficient energy range and resolution to determine the continuum shape¹. The continuum shape has usually been attributed to optically thin thermal bremsstrahlung, consistent with earlier results for the 1972 14 May burst² and the Apollo burst³. In addition, low-energy absorption features (identified as cyclotron lines) and high-energy emission features (identified as redshifted annihilation lines) have been reported¹. These spectral features argue strongly that γ -ray bursts originate from highly magnetized neutron stars¹.

However, there are several difficulties with the above interpretations. First, the interpretation of the continuum as optically thin thermal bremsstrahlung implies a very large emitting volume (especially if the emitting region is homogeneous and if the source is further than 10 pc) whereas the cyclotron lines imply a small volume⁴. Second, various theories⁵⁻⁷ suggest that the burst originates in a small volume near the surface of a neutron star rather than from a dilute, 100-km sphere implied by thermal bremsstrahlung. Third, thermal bremsstrahlung does not always fit the observations; often the observed spectrum are so hard that no thermal bremsstrahlung spectrum can fit the data. Finally, the cyclotron lines reported to date may be instrumental artefacts⁴.

We have investigated the spectral distribution emerging from a plasma at temperatures typical of γ -ray burst spectra ($kT > 100$ keV) but where there is an underlying source of photons which are reprocessed by inverse Compton scattering. As the free-free cross-section is much smaller than the Compton cross-section, the overlying plasma will have negligible thermal emission. We use a Monte Carlo method^{8,9} of calculation rather than the Fokker-Planck method¹⁰⁻¹² because the Fokker-Planck equations break down when the temperature

is >100 keV and/or when there are large-changes in energy occurring in only a few scatterings¹³. The physics of our model is similar to the two-temperature accretion disk model¹⁴ suggested for Cygnus X-1. The primary difference from previous (X-ray) Monte Carlo calculations is that we treat rigorously the coupling between the maxwellian electron distribution and the Compton cross-section to ensure that both high-temperature effects and the angular distribution are accounted for. The angular distribution of the electron involved in the scattering can have deviation from isotropy due to the angular dependence of the cross-section.

To include temperature and angular effects, the Compton cross-section must be weighted by an isotropic maxwellian electron distribution. The total Compton cross-section for a photon of energy E_p into a plasma of temperature T_e and unit density is

$$\sigma(E_p, T_e) = 2\pi \int_0^\infty \int_0^\pi N(E_e, T_e) \sigma(E_p, E_e, \alpha) \sin \alpha \, d\alpha \, dE_e \quad (1)$$

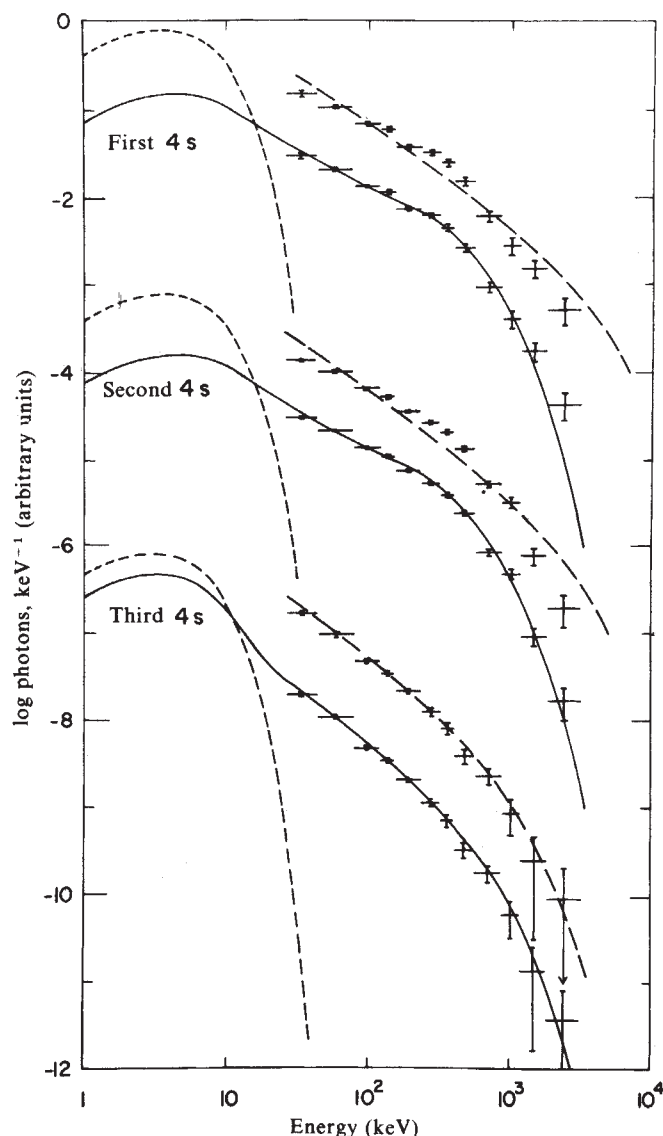


Fig. 1 Best-fit thermal bremsstrahlung spectra and comptonized black-body spectra for GB781104. The short-dashed curves are the initial X-ray black bodies and the solid curves are the resulting comptonized spectra. The long-dashed curves are the best-fit thermal bremsstrahlung spectra. The data points (repeated for the thermal bremsstrahlung and comptonized black-body fits) are from the Berkeley/Los Alamos ISEE 3 solar X-ray spectrometer.

where the relativistic maxwellian is

$$N(E_e, T_e) dE_e \propto (E_e + E_0)(E_e^2 + 2E_0E_e)^{1/2} \exp(-E_e/kT_e) dE_e \quad (2)$$

with $E_0 = 511$ keV and $\sigma(E_p, E_e, \alpha)$ is the Compton cross-section for a photon of energy E_p onto the electrons of kinetic energy E_e moving at an angle of α with respect to the photon. To determine $\sigma(E_p, E_e, \alpha)$, a Lorentz transformation is made to a frame in which the electrons are at rest and then the cross-section is found by the Klein-Nishina formula⁸. That is, $\sigma(E_p, E_e, \alpha) = (1 - \beta \cos \alpha) \sigma_{KN} [E_p \gamma (1 - \beta \cos \alpha)]$ where $\gamma = 1 + E_e/E_0$, $\beta = (1 - \gamma^{-2})^{1/2}$ and σ_{KN} is the Klein-Nishina cross-section.

For this preliminary study, we assumed that the emitting region consisted of a point source of black-body radiation at the centre of a spherical volume containing a hot thermal plasma with no magnetic field. Three parameters were chosen to characterize the region: T_{BB} , the black-body temperature of the central source; ρx , the column density (that is, the radius times the electron density); and T_e , the temperature of the comptonizing volume. The following steps were taken to track each photon:

(1) A random number, r_1 , between 0.0 and 1.0 is selected and used to determine the distance the photon travels before undergoing a Compton interaction:

$$d = -(N_0 \sigma(E_p, T_e))^{-1} \ln(r_1) \quad (3)$$

where N_0 is the electron density of the plasma. If the location of the proposed Compton scattering site is outside the assumed size of the volume, the photon is considered to have escaped and the emerging energy spectrum is incremented at the photon's energy.

(2) Once a location for the Compton scattering is known, the energy of the electron involved is found by solving for E_e in

$$r_2 = \frac{1}{\sigma(E_p, T_e)} \int_0^{E_e} \int_0^\pi N(E, T_e) \sigma(E_p, E, \alpha) \sin \alpha d\alpha dE \quad (4)$$

where r_2 is a new random number.

(3) The angle between the photon and the electron is found by solving for α_e in

$$r_3 = \int_0^{\alpha_e} \sigma(E_p, E_e, \alpha) \sin \alpha d\alpha / \int_0^\pi \sigma(E_p, E_e, \alpha) \sin \alpha d\alpha \quad (5)$$

The other angle to complete the definition of the photon's direction relative to the electron's is found simply as $2\pi r_4$.

(4) Knowing E_p , E_e , and α_e allows a Lorentz transformation to the rest frame of the electron where the Compton interaction is calculated. Two additional random numbers, coupled with the formulae for the radiation pattern of Compton scattering on an electron at rest, give the scattering angle and the change in energy of the photon in the electron rest frame. The inverse Lorentz transformation gives the new energy and direction of the photon after the Compton collision in the laboratory (that

is real world) frame. Steps (1) to (4) are repeated until the photon escapes.

The results of the Monte Carlo calculations have been compared directly with the observed spectrum of the typical and well studied burst GB781104 (refs 1, 4, 15, 16). It was not computationally feasible to perform a Monte Carlo calculation at each combination of T_e and ρx required to determine the best-fit parameters and their errors. Rather, calculations were performed on a grid of T_e and ρx values which were then interpolated to obtain a spectrum for a particular set of T_e and ρx . Table 1 gives the best fit for both thermal bremsstrahlung and comptonized black bodies with their joint 68% confidence levels (calculated by the method of ref. 17) for three successive 4-s intervals. In Table 1, χ^2_c , N_{df} , T_{TB} , χ^2_{TB} are, respectively, the χ^2 of the comptonized black body fit, the number of degrees of freedom, the best-fit thermal bremsstrahlung temperature, and the χ^2 for the thermal bremsstrahlung fit. Figure 1 shows, for each 4 s interval, the initial black body as a short-dashed curve, the resulting comptonized spectrum as a solid curve with ISEE 3 solar X-ray spectrometer data (with 1 σ error bars), and the best-fit thermal bremsstrahlung spectrum (long-dashed curve) with the ISEE 3 data. The reconstructed spectrum obtained by unfolding the observed counts depends on the assumed spectral shape. For this reason, the unfolded ISEE 3 data points differ slightly depending on whether thermal bremsstrahlung or a comptonized black body is assumed. For clarity, the thermal bremsstrahlung fit (with unfolded ISEE 3 data points) is offset from the comptonized black body fit (and its data) in Fig. 1. The optically thin thermal bremsstrahlung formula included the relativistic Guant factor of ref. 18.

Optically thin thermal bremsstrahlung could not satisfactorily fit the data during either of the first two 4-s intervals due to the relative hardness of the spectrum. Only extremely high temperature thermal bremsstrahlung came close to fitting the data. In contrast, the comptonized spectrum had a much more reasonable plasma temperature ($T_e = 155$ keV compared with $T_{TB} = 3,800$ keV) and a much lower value of χ^2 (for example, 19.0 for the comptonized black body compared with 140.0 for thermal bremsstrahlung). (The thermal bremsstrahlung fit is not substantially improved even if a broad emission line between 230 and 600 keV is assumed; T_{TB} is reduced to 3,300 keV with $\chi^2_{TB} \sim 80$.) Thermal bremsstrahlung would not fit our data in the second interval either, giving a χ^2_{TB} of 330 whereas the comptonization model gives a χ^2_c of 18.6. These values of χ^2_c , although much better than those for thermal bremsstrahlung, are marginally too high to be considered statistically acceptable. However, the spectrum is changing on a time scale shorter than our sampling rate (4 s), which might explain these rather large values of χ^2 . The third 4-s interval could be fit by either a thermal bremsstrahlung spectrum (with $\chi^2_{TB} = 10.7$) or a comptonized black body (with $\chi^2_c = 8.8$).

Note that, in all three intervals, the best-fit black body has an X-ray temperature of ~ 2 keV. The χ^2 surfaces are not quadratic functions of the parameters and, thus, one cannot estimate from the 68% confidence level the parameter limits for other confidence levels. The term in Table 1, $kT_{BB,95\%}$, gives the lowest black body temperature that fits the data at the 95% confidence level. Although the first 4-s interval could be consistent with a very cool source of photons, the next two intervals are only consistent with black-body temperatures > 0.4 keV. There is no evidence to indicate that the underlying X-ray source is any known type of X-ray activity, in particular, a burster. Rather, the X-ray source is probably a result of heating of the surface by the overlying, hotter region.

One potential difficulty with our model is that the cooling time of the overlying plasma is $\sim 10^{-6}$ s, much shorter than the duration of the bursts. Thus, some process must replenish the overlying region. A similar problem is also found in most other γ -ray burst models⁷.

Three of the assumptions we have made are: (1) the source is at the centre of a sphere; (2) the emerging spectrum can be found by interpolation between Monte Carlo calculations; and

Table 1 Best-fit parameters for GB781104

	First 4 s	Second 4 s	Third 4 s
$\chi^2_{TB} (N_{df} = 10)$	140	330	10.7
kT_{TB} (keV)	3,800	2,334	786 ± 70
$\chi^2_c (N_{df} = 8)$	19.0	18.6	8.8
kT_e (keV)	155 ± 35	160 ± 20	$240^{+\infty}_{-70}$
$\rho x (10^{24} \text{ e cm}^{-2})$	4.5 ± 1.2	4.0 ± 0.6	1.2 ± 1.2
kT_{BB} (keV)	$2.4^{+9.6}_{-2.4}$	2.4 ± 1.5	2.0 ± 0.4
$kT_{BB,95\%}$ (keV)	0.0	0.4	0.7

(3) there is no magnetic field. Preliminary calculations indicate that the first two assumptions do not cause large changes in the shape of the resulting spectrum, although changes in the best-fit parameters could occur. Recently, very high magnetic fields ($\sim 10^{12}$ G) have been assumed in γ -ray burst models⁵⁻⁷. However, we have made a preliminary comparison of the spectral shapes predicted for cyclotron processes (see ref. 7) with γ -ray burst spectra. Cyclotron spectra seem to be much softer than typical γ -ray burst spectra. A very hard spectrum (such as GB781104) would be even more difficult to fit. To prevent cyclotron processes from dominating the radiation transport, the magnetic field in the comptonizing region must be $\leq 10^9$ G. Even a field as low as 10^9 G would be able to confine the plasma on the time scale of replenishment.

There are two other features of some γ -ray burst observations which could naturally be explained by comptonized X-ray models. In some bursts, the spectrum consists of a normal γ -ray component plus a soft component (with $kT < 20$ keV)¹. The soft component is discernible during periods when the γ -ray flux above 100 keV has dropped to near background. Two examples of such bursts are GB790329 and GB790524 (see Fig. 2 in ref. 1). The Comptonized X-ray burst model would give a similar two-component spectrum when the density of the overlying region decreases so that some low-energy photons escape without having scattered, and thus, retain the spectrum of the X-ray black body. Those photons that do scatter produce the normally observed γ -ray component.

The second observation which might be explained by the comptonized X-ray model concerns the γ -ray burst of 19 November 1978. The spectrum initially had a low energy cutoff starting at ~ 400 keV; as the spectrum evolved, the cutoff moved to lower energies resulting in a monotonic-appearing spectrum^{19,20}. This behaviour is consistent with a comptonized X-ray model if the density of the scattering medium was high. At high densities, there is a sufficient number of scatterings to equilibrate the photons, thus producing a Wien peak. If the density decreases, the spectrum would become monotonic.

Most of the annihilation lines¹ are probably too narrow to be explained by Wien peaks. In fact, the broad emission feature in 19 November 1978 was not identified as an annihilation line by Mazets *et al.*¹ Mazets *et al.*¹ claim an annihilation line in 4 November 1978 whereas our comptonized black body fits do not require such a line. However, a smooth line through our data points in the thermal bremsstrahlung unfolding during the first 4 s would seem to require a line at about the intensity and shape reported by Mazets *et al.*¹ (see Fig. 1). This demonstrates how spurious line features can be produced if a wrong continuum shape is assumed.

The best-fit parameters indicate that γ -ray burst continua are consistent with an X-ray source which undergoes inverse comptonization by a hot overlying plasma. Such a hot plasma is normally assumed for a γ -ray burst and we offer no explanation of why the hot plasma would be overlying an X-ray source. Rather, these results indicate that a particular continuum shape other than thermal bremsstrahlung can sometimes better fit the available data. The implications of these fits should be explored with more detailed theoretical work.

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Metal-organic coating interface as studied by emission Mössbauer spectroscopy

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A major unknown in an understanding of the interface between an organic coating and a metal substrate is the chemical nature of the interfacial region. This is specially important in understanding the phenomena that control 'wet adhesion', the ability of coated metals to withstand long exposure to high humidity conditions, and the disbonding of light-sensitive polymers used in lithographic plates and photoresists. We report here how ^{57}Co emission Mössbauer spectroscopy can be used to study the chemistry of the interfacial region in a model system consisting of cobalt metal and a commercial polybutadiene coating.

A previous study showed that the Mössbauer technique was suitable for studying corrosion beneath a coating². Cobalt is selected here as the substrate metal because the isotope ^{57}Co is Mössbauer-active and can be concentrated at the metal surface using electrodeposition. Polybutadiene is selected as the coating because of our previous work with this material³ and the utilization of polybutadiene by Dickie and co-workers⁴ and by Castle and Watts⁵ in corrosion studies.

Emission Mössbauer spectroscopy is based on the 14.4-keV γ ray that is emitted from ^{57}Fe as a consequence of the electron capture decay of ^{57}Co . Although the Mössbauer spectra obtained with this method are of ^{57}Fe , it is likely that it will be possible to obtain information about the chemical state of the initial ^{57}Co isotope. Indeed, in many cases emission spectra of cobalt compounds give hyperfine parameters expected on the basis of known oxidation state, structure and magnetic properties. It is also commonly observed that a fraction of the oxidation states of ^{57}Fe is atypical of the original oxidation state of ^{57}Co . These atypical oxidation states are produced as a consequence of the Auger cascade that follows the electron capture decay of ^{57}Co (refs 6-8). The fraction of these states that are stabilized is a function of chemical environment. For example, no atypical ^{57}Fe oxidation states are found in metallic environments, whereas in other compounds, such as hydrated cobaltous salts, a significant fraction of Fe^{3+} is found. Our interpretation of the emission Mössbauer data is based on extensive emission studies of the oxides, hydroxides and hydroxyoxides of cobalt that may be present at a cobalt metal-polymer interface⁹⁻¹¹. The reported changes in the emission spectra of cobalt at this interface represent either direct changes in the chemical state of cobalt or changes in the environment of cobalt that in turn affects the stabilization of atypical ^{57}Fe charge states.

Cobalt, doped with ^{57}Co , was electrodeposited at room temperature on a cobalt substrate at a current density of 0.01 A cm^{-2} for 5-20 s while the metal was immersed in a bath

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Table 1 Mössbauer spectra parameters

Sample spectrum of electrodeposit	Component	δ (mm s ⁻¹)	Δ (mm s ⁻¹)	$H_{5/2}$ (kOe)	Γ (mm s ⁻¹)	Area of component (mm s ⁻¹)	A (%)
Before applying the polybutadiene coating (Fig. 1)	Fe ³⁺	-0.093	0.808	338	0.996	0.284	71
	Fe ²⁺	-0.952	2.248		0.540	0.016	4
	Fe ⁰	0.398	0.124		0.885	0.097	24
After applying polybutadiene coating but before baking	Fe ³⁺	-0.149	0.792	336	1.026	0.277	67
	Fe ²⁺	-0.925	2.096		0.786	0.020	5
	Fe ⁰	0.532	0.168		1.241	0.114	28
After baking the polybutadiene coating for 30 min at 200 °C (Fig. 2)	Fe ³⁺	-0.127	0.701	337	1.032	0.155	38
	Fe ²⁺	-0.883	2.214		1.216	0.153	38
	Fe ⁰	0.380	0.220		1.088	0.096	24

Chemical shift (δ) is given with reference to an enriched K₄Fe(CN)₆ absorber; Δ = quadrupole splitting value; $H_{5/2}$ = hyperfine magnetic splitting; Γ = line width; A = relative amount of component.

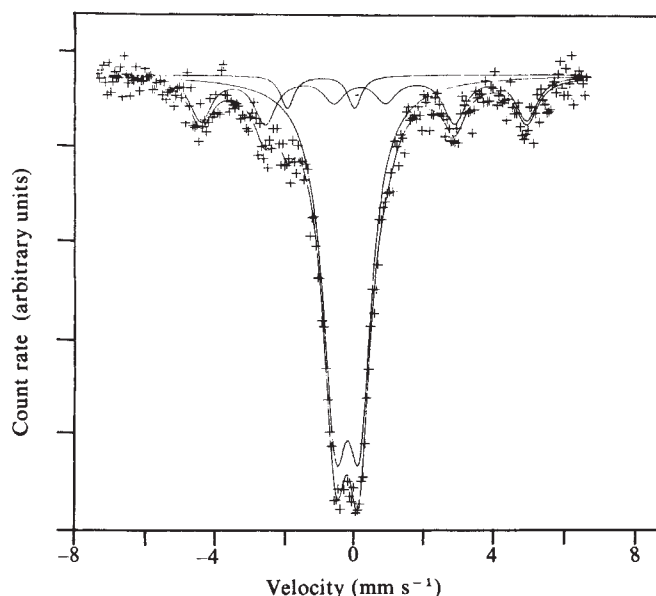


Fig. 1 ⁵⁷Co Mössbauer emission spectrum of a thin cobalt electrodeposit used as the starting point in the experiment described in the text.

containing 0.31 g l⁻¹ CoSO₄·7H₂O, 100 g l⁻¹ MgSO₄·7H₂O, and 30 g l⁻¹ H₃BO₃, pH = 4.0. The calculated thickness of the deposit was 20–120 nm assuming a current efficiency of 100%, but the actual thickness was considerably less. The Mössbauer spectrum of a thin deposit is shown in Fig. 1 in which the metallic component (six-line spectrum) was ~24% and the ionic component was ~76%. These values are calculated on the basis of the area under the curve for each component and assume that the recoilless fraction is the same for all components. The ionic component presumably results from superficial oxidation of the metallic surface on exposure to air. The ratio of the metallic to the ionic component increased as the thickness of the electrodeposit was increased. Maximum sensitivity in the experiments to be described was obtained with deposits which yielded a spectrum similar to that in Fig. 1. The major doublet is at a position characteristic of Fe³⁺ and only a small amount of Fe²⁺ (4%) is revealed by computer curve fitting. On the basis of the studies of Barr¹² and of Rice *et al.*¹³, it is likely that the outer layer of cobalt exposed to the atmosphere has a composition equivalent to CoOOH. Identification of the surface composition by Mössbauer techniques alone does not appear practical as Co(OH)₂ exhibits spectra showing different amounts of Fe²⁺ and Fe³⁺ depending on the method of preparation¹.

The cobalt surface was then coated with a commercial polybutadiene coating (Budium RK-662, DuPont) to a thickness of ~15 µm and the emission spectrum was again obtained without baking the coating. The spectrum was very similar to

that shown in Fig. 1. The Mössbauer parameters are given in Table 1. The coated metal was cured by baking at 200 °C in air for 30 min and a spectrum was again obtained. As shown in Fig. 2 and in Table 1, the Fe²⁺ doublet is now a substantial part of the total spectrum. Two possible changes at the interface could account for the increase in the Fe²⁺ component. First, an interaction between the cobalt oxide and the polymer may have reduced Co³⁺ in the surface oxide to Co²⁺. Second, the treatment may have led to the dehydration of a Co²⁺ oxide with the result of destabilization of Fe³⁺. (Stabilization of Fe³⁺ in the presence of water is thought to be the result of oxidation of Fe²⁺ to Fe³⁺ by OH radicals produced by the radiolysis of water molecules by the Auger electron ionization⁶⁻⁸.) The reduction of the surface oxide is supported by the tentative conclusion of Castle and Watts⁵ that the oxide on iron is partially reduced during the baking of polybutadiene coating on steel. The latter conclusion was tentative since it was based on X-ray photoelectron sputtering studies of stripped oxides and it is well known that the sputtering process may lead to changes in valence states of the cations in oxides¹⁴.

The experiments described in Table 1 were repeated several times with similar results showing that the Mössbauer technique is sensitive at the level required to give information about the chemical nature of the organic coating–metal interface. The following conclusions can be drawn: (1) The contact between the cobalt metal and the polybutadiene is through an oxide or hydrous oxide at the interface. (2) No major changes at the cobalt interface occurred after application of the coating and

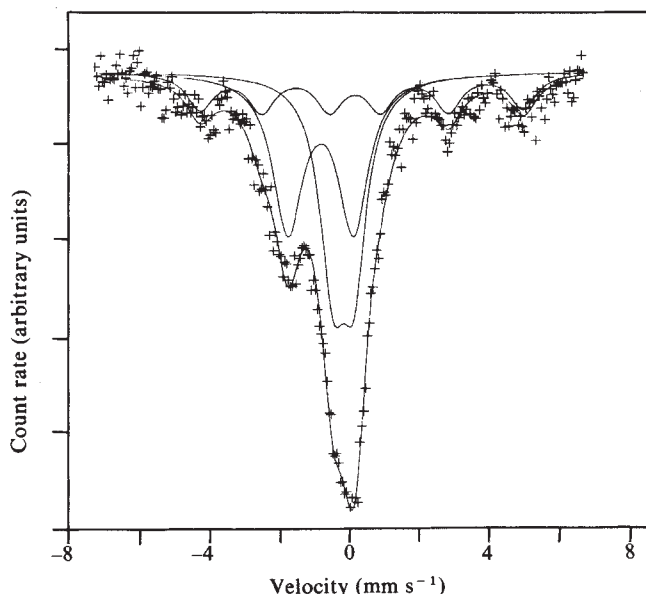


Fig. 2 ⁵⁷Co Mössbauer emission spectrum of sample after coating with polybutadiene and baking at 200 °C for 30 min. Compare spectrum with that in Fig. 1.

before thermal treatment. (3) The oxide on cobalt does not thicken during the baking process. (4) Thermal treatment at 200 °C causes a significant amount of conversion of Fe^{3+} to Fe^{2+} at the oxide-coating interface. (5) The reduction process cannot be a consequence of the reaction, $2\text{Co}^{3+} + \text{Co} = 3\text{Co}^{2+}$, during baking of the coating because the relative amount of unoxidized metal remains approximately constant before and after baking. (6) The conversion of Fe^{3+} to Fe^{2+} may be attributed to either a reduction of Co^{3+} to Co^{2+} or to the destabilization of Fe^{3+} by the dehydration of Co^{2+} oxide.

Small changes in the chemical shift and in the quadrupole splitting values before and after baking the polybutadiene may be significant, but we prefer to wait for further experiments with other metal-polymer coating systems before drawing further conclusions.

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Tabular icebergs in ocean waves

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Ocean waves are a principal agent in the deterioration and ultimate breakup of Antarctic tabular icebergs in the Southern Ocean. We present here some preliminary results from a recent (January 1982) field season on board HMS *Endurance* during which two tabular icebergs of very different shape were studied in detail. The data augment the results of a similar season of work^{1,2} which took place in 1981 near the South Sandwich and South Orkney Islands. Instruments to measure rigid body motions and strain gauges to determine wave-induced bending were deployed at the surface centre of the two icebergs visited, while a Waverider buoy simultaneously monitored the ocean wave-energy spectrum. The precise underwater shape of each iceberg was determined by flying a 60-MHz radar (developed for use over land ice)^{3,4} in a grid pattern across the surface of the iceberg. By this means a complete three-dimensional picture of the iceberg could be inferred. The results show that icebergs tend to act as low-pass filters and inhibit short period waves. Furthermore, they selectively resonate at certain wave periods; the strain data, in particular, indicate unexpectedly large flexure which cannot be explained by simple bending alone. Finally, the geometry of icebergs can be such as to render them unstable and liable to turn over.

The first iceberg, which was encountered close to Clarence Island in the South Shetlands, was about 1,200 m long by 480 m wide with a freeboard varying between 39 and 57 m. Radio echo profiles showed the iceberg to be roughly trapezoidal in cross-section with the bottom sloping along its length. The second iceberg was located further north at ~62°51.3'S,

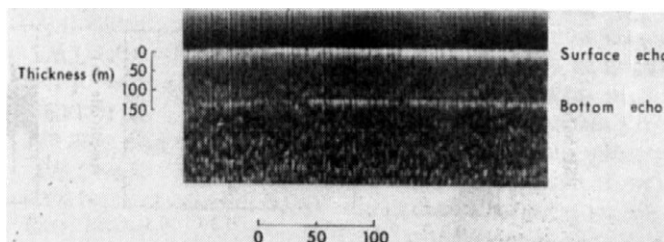


Fig. 1 Radio echo sounding profile showing upper and lower surfaces and calculated scales.

53°31.7'W. It was much larger (3,630 × 1,740 m), but had a freeboard of only 20-28 m. The underside was approximately flat with no evidence of bottom crevassing. Figure 1 represents a longitudinal radio echo transect for this iceberg showing the position of the surface and bottom echoes. A feature of Fig. 1, which has been found in many of our radar profiles, is the region of weak bottom return echo to the left of the centre of the figure. This phenomenon is possibly due to a refrozen crevasse where the new ice contains seawater salts of relatively higher dielectric absorption.

The overall experimental plan benefited considerably from the experiment which had taken place the previous year. Two Royal Navy Wasp helicopters were used to ferry instruments and personnel onto the surface of the ice, and to carry out the radio echo sounding. On the surface, three accelerometers mounted on gimbals were used to measure heave, surge and sway, and two tiltmeters measured pitch and roll. Three 1-m deep trenches in a 120° rosette configuration^{5,6} were dug for the strain measuring equipment. The three strainmeters had been developed by the Scott Polar Research Institute (SPRI) specifically for use on sea ice⁷. Their use on an iceberg surface presented serious mounting problems as the top 30 m are made up of firn and snow interspersed with ice layers. A satisfactory mount was finally achieved by driving 2-m long aluminium cylinders into the surface and then rigidly fixing the strainmeters to them. Simultaneous measurements of the wave field in the waters around the berg were collected using a Waverider buoy located several kilometres away.

The main features of the icebergs' motions can be inferred from Fig. 2, where the original time series, and the unsmoothed and smoothed power spectra are presented for the larger iceberg. As might be expected, the bandwidth of the Waverider buoy data is much greater than that of any of the sensors located on the iceberg itself. This demonstrates that icebergs act to filter out the shorter wave periods found in the forcing spectrum. The 8-s cutoff is very distinct for surge, roll and strain, though the iceberg shows energy in heave at periods as low as 6 s. It is also significant that the dominant peak in the Waverider spectrum does not necessarily occur at the same period as those in the other spectra. In heave the peak occurs at about 16 s, in surge at 10 s, and in roll at 11 s. This agrees with rough calculations to find the respective natural frequencies, and indicates that the iceberg can tune to the incident ocean waves and resonate at a period which is dependent on its geometry and structure. This is especially apparent in the strain spectrum which shows three distinct peaks at periods of 10, 15 and 50 s, showing that the iceberg is extracting energy from the ocean waves at selective periods. The strains corresponding to these peaks are of the order of 10^{-6} , considerably greater than we would expect unless a resonance was occurring (10^{-6} is typical of sea ice floes two orders of magnitude thinner⁸).

Figure 3, showing another startling example of resonance seen on the first iceberg, is a copy of the roll data as recorded on the iceberg during the experiment. Dominant periods in the data are clear at about 14 and 100 s. The shorter of these oscillations represents the ocean wave forcing, the longer is the natural period of roll for an iceberg of this shape and structure. It is interesting to speculate about the origin of the 100-s roll. The oscillation might have been generated impulsively or be continually maintained by some energy in the open water at

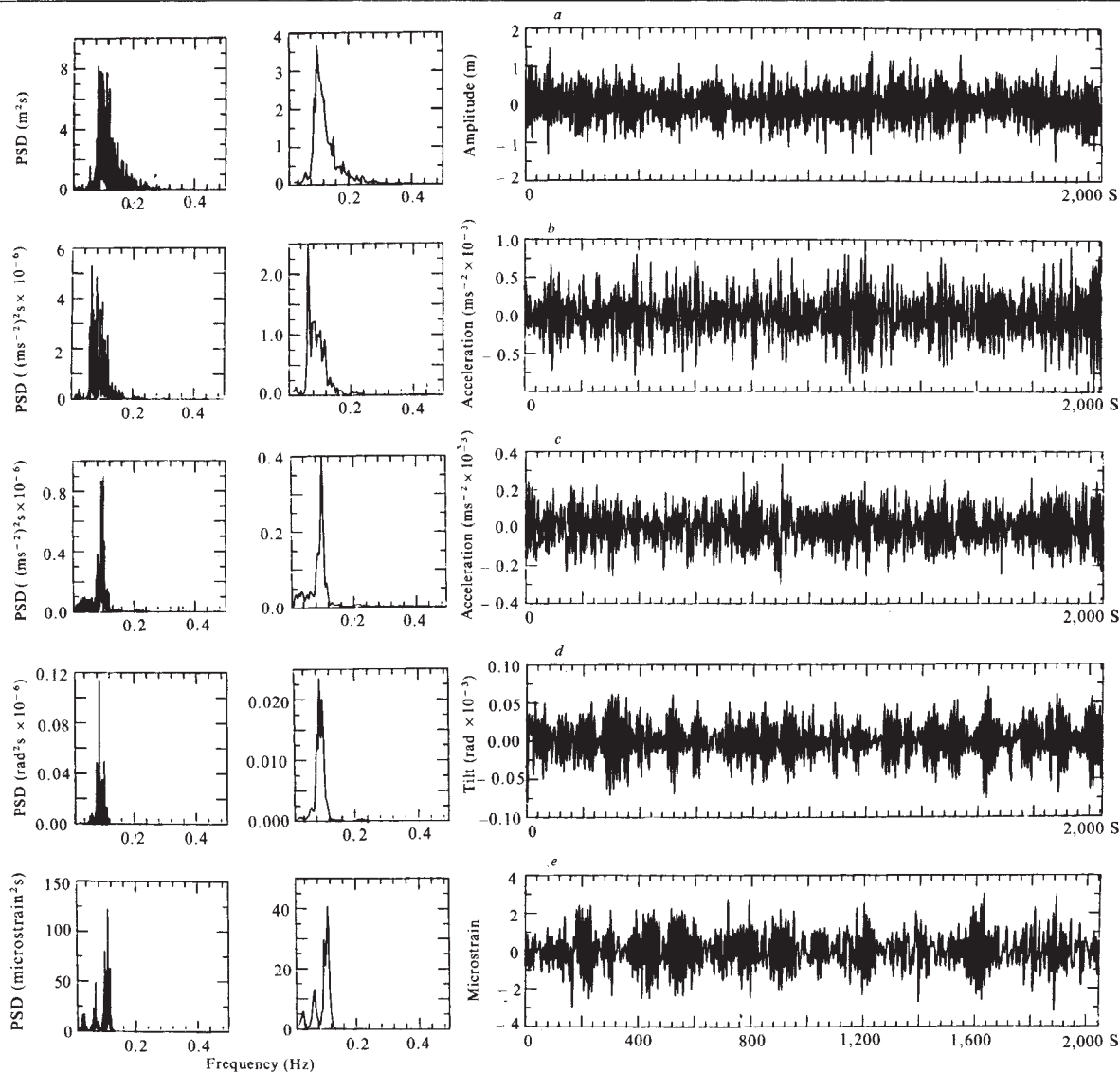


Fig. 2 Time series and computed power spectra for *a*, the Waverider buoy data and *b*, the heave; *c*, surge; *d*, roll; and *e*, strain motions of a tabular iceberg. The smoothed spectra are grouped by nine continuous power spectral density (PSD) values.

that period. In the first case the motion would decay at a rate determined by the 'Q' of the ice-water system. In the second, the energy might come from wave packets forcing fluid motions on the length scale of the modulation envelope.

A simple calculation drawn from Lewis' review on the motion of ships in waves⁹ may be used to interpret the 100-s roll. Figure 4 gives the necessary parameters for the calculation. The metacentric height, GM , for the iceberg is given by

$$GM = \frac{a^2}{3d} - l + \frac{d}{2} \quad (1)$$

The expression given by Lewis for the natural period of roll is

$$T^2 = \frac{4\pi^2 r^2}{g GM} \quad (2)$$

where r is the effective radius of gyration about the axis of roll which includes a contribution from the added inertia of the fluid entrained by the rolling iceberg, and g is the acceleration

due to gravity. With appropriate values for the width (480 m), the thickness (273 m) and the density (850 kg m^{-3} —assumed constant but reduced because of any density variations in the berg), the effective radius of gyration is found to be nearly 400 m. This is more than double the value for the iceberg if the fluid's contribution is neglected, and emphasizes that added inertia must be taken into account if reliable theoretical estimates of rolling period are to be found. Finally, it is of interest to investigate the stability of the rolling iceberg. Adopting the suggestion of Weeks and Mellor¹⁰ that an iceberg should be considered unstable if its metacentric height is <10% of its width, we find that the iceberg was just stable.

In conclusion, it must be stressed that there is a singular lack of data on the behaviour of icebergs in ocean waves. Two field seasons have been carried out by SPRI in cooperation with Norsk Polarinstitutt, and Foldvik *et al.*¹¹ have reported some measurements which took place during the Norwegian Antarctic Research Expedition of 1978–79. The results from the two SPRI seasons together constitute a unique data set which may

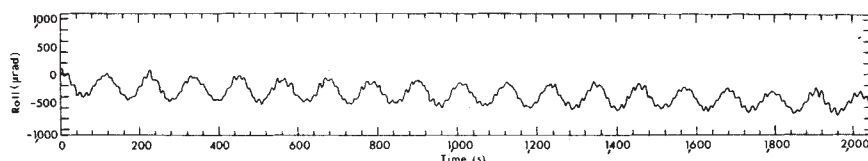


Fig. 3 Roll data record from first iceberg.

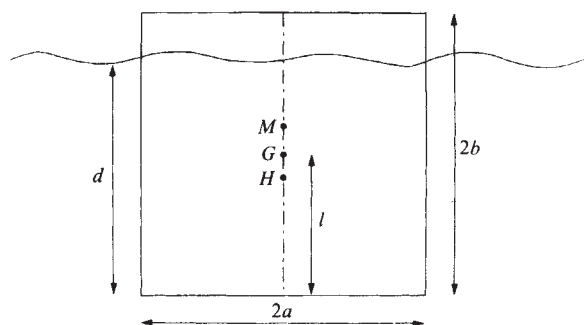


Fig. 4 Simplified rectangular iceberg section. *M* is the iceberg's metacentre, *G* is its centre of mass, and *H* is its centre of buoyancy (the centre of mass of the displaced fluid).

be used to predict sea-keeping characteristics for tabular icebergs generally. Foldvik's discussion is unfortunately limited to tilt alone as his strain measuring system was unable to resolve the slight cyclic bending which occurred. In presenting his tilt data, Foldvik has derived theoretical curves based on equation (2) but has neglected added inertia. We have shown that this can seriously underestimate any natural roll period calculations. The data reported here fall at the two extremes of his measurements.

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A deep seismic reflection profile over a Caledonian granite in central England

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In 1980, a short, deep reflection profile, undertaken jointly by the University of Leicester and the Institute of Geological Sciences, crossed a buried Caledonian granite to the south-west of Melton Mowbray in Leicestershire. Little structure is seen in the section between the top of the granite at 0.25 s two-way time (s TWT), and a zone of very strong sub-horizontal reflections at 6 s TWT. A further reflection zone is identified at 7.2 s TWT below which there are a series of strong but impersistent reflections to the full recording time of 12 s TWT.

The data indicate that the granite must be relatively homogeneous and underlain by several marked sub-horizontal boundaries at mid- to lower-crustal levels. This suggests that, during emplacement, either the granite must have had little effect on the lower crust sampled by the profile, or the lower crust was mobile enough to have developed layering since the granite was emplaced during the Caledonian orogeny.

The various granitic rocks of Leicestershire observed at Mountsorrel, Countesthorpe, Croft, Enderby, Stoney Stanton and in the recent NCB borehole at Kirby Lane, south-west of Melton Mowbray (Fig. 1) are considered to be the surface expression of a large calc-alkaline granitic pluton^{1,2}. These granitic rocks generally have a strong magnetic signature and have been shown to be distinguishable from the surrounding Precambrian, meta-sedimentary and meta-volcanic rocks at basement level by their near surface, lower P-wave seismic velocity, derived from seismic refraction data³.

A particularly suitable problem for a limited-budget, deep seismic reflection programme was to examine whether a granite may provide a transparent medium for acoustic energy to penetrate the lower crust. It was therefore decided that a survey should be undertaken over the large, 250 nT, aeromagnetic anomaly associated with the Melton Mowbray granite, in particular along a disused railway line passing within 1 km of the Kirby Lane borehole.

Data acquisition was carried out using the following parameters: 9 kg explosive charges at 15 m depth, recorded by a 24 channel 'end-on' array: 12 geophones per station at a 50 m station interval. Shooting every station resulted in 12-fold multiplicity, horizontal subsurface cover being 2.125 km. Data were recorded to 12 s TWT and processed at a 4-ms sample rate, using a routine oil industry processing suite (Fig. 2).

At first sight the section is divided into three distinct intervals: (1) from the surface down to a fairly complex but strong reflector between 0.25 and 0.32 s TWT; (2) a relatively signal-free zone down to a strong band of reflections at 6 s TWT; and (3) down to the full recording time including many strong but impersistent phases. The complete record is generally laterally homogeneous except for a small region between 1.6 and 1.9 s TWT in the signal-free interval, which includes a few high frequency continuous reflections at the north end of the profile. These reflections are associated with anomalously high stacking velocities. Throughout the section, although most of the reflections are almost horizontal, some do show dips up to a maximum of ~100 ms in 2 km, giving maximum apparent dips of ~10°.

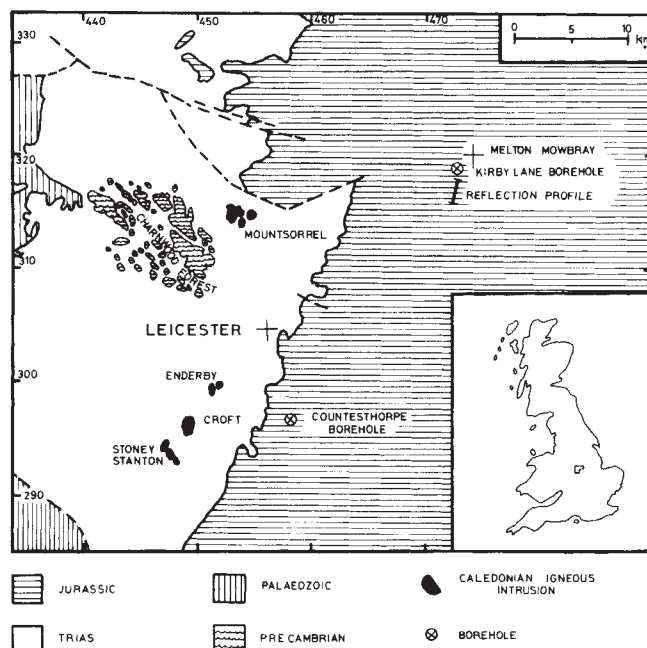


Fig. 1 Location map.

To examine the variation of reflection continuity with depth, a plot of length of continuous, correlatable signal (normalized to the maximum observed length averaged over a 0.2 s window) versus two-way time was produced (Fig. 3).

The anomalous high-frequency reflections between 1.6 and 1.9 s TWT were omitted being of very short lateral extent, and therefore uncharacteristic of the whole profile. The two minor peaks at ~0.6 and 0.9 s TWT are almost certainly multiples. The signal-free zone is divided into an upper, completely free zone down to 4 s TWT and a lower zone including a few, short, continuous correlatable pulses. Below the 6 s TWT band of reflections a second prominent group at 7.2 s TWT appears at the top of the broad zone of strong but impersistent reflections. To estimate the depth of the various reflection zones, a velocity-depth profile has been derived from other data.

The granite velocity, derived from Birch⁴ is compatible with that observed near the upper surface of the Mountsorrel granodiorite⁵. Lower crustal and sub-Moho velocities, together with a reasonable depth to the Moho of 30 km, were obtained from the LISPB results⁶, which are not inconsistent with a possible velocity structure beneath Charnwood Forest⁷ (Fig. 1). Using these velocities it is necessary to invoke a change from 6.3 to 7.1 km s⁻¹ at mid-crustal levels. It is considered that such a change could occur at the major reflection band at 6 s TWT. Although the velocity jump and associated two-way time are purely speculative, there is supporting evidence in the fact that interval velocities derived from the stacking velocities of the three most prominent reflections at 0.25, 6.0 and 7.2 s TWT are very similar to the constructed velocity-depth profile (Fig. 4). This has been used to generate the depth scale on Fig. 3. Below 7.2 s TWT, the identification of any coherent events as primary reflectors would be highly speculative, particularly in view of the very limited horizontal subsurface cover.

There are several more conclusions to be drawn from this study. The Leicestershire granite pluton does provide an

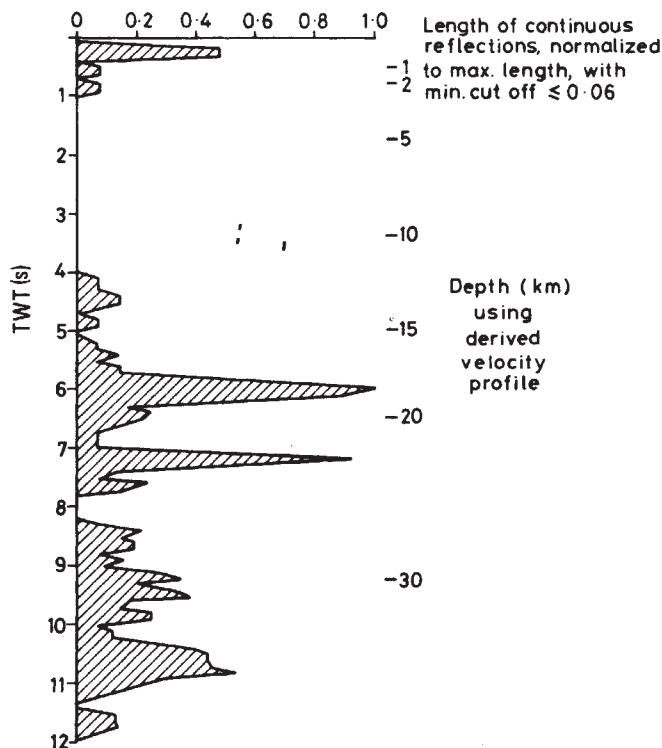


Fig. 3 Section reflection character.

acoustically transparent medium through which seismic energy can penetrate the lower crust. However, it is not possible to identify positively the base of granite. A slight change in character of the section below 4 s TWT may indicate a different crustal fabric but the main change occurs below 6 s TWT. The Moho, at a depth of ~30 km, almost certainly lies within the broad zone of deep reflections below the two prominent reflection bands at 6 and 7.2 s TWT. Although it is unreasonable to identify primary reflectors in this broad zone, and therefore unreasonable to speculate on the precise nature of the crustal-upper mantle transition, the presence of the deep reflections does imply that there are changes in acoustic impedance over a broad depth zone extending into the upper mantle, in contrast to the uniform structure apparent in the upper part of the section below the top surface of the granite.

S ← 2.125 km → N (s TWT)

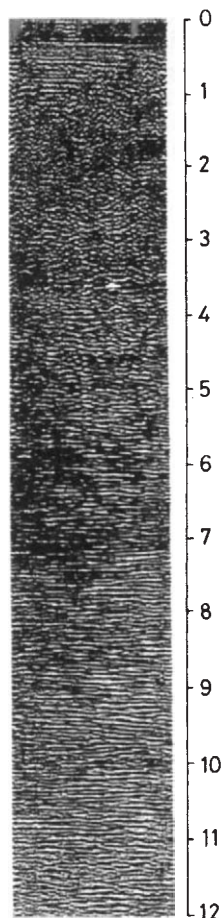


Fig. 2 Seismic section.

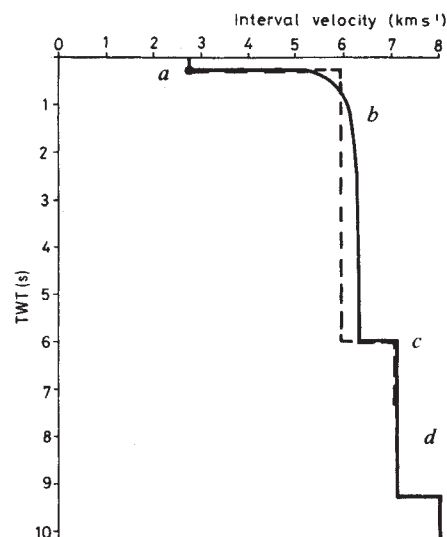


Fig. 4 Velocity-depth profile. ---, Velocities from V_s for prominent reflections only. —, Velocity profile used to convert from time to depth, derived from: a, borehole log; b, granite; c, break occurring at prominent reflector at 6.0 s TWT; d, LISPB and study of P-wave delay between Charnwood and Eskdalemuir.

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Methane flux in the Great Dismal Swamp

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Methane is an important component of the biogeochemical cycle of carbon with potentially critical roles in both atmospheric chemical and radiation transfer processes^{1–4}. Limited evidence is available which suggests an increase in global tropospheric methane during the last decade^{5,6}. To understand and assess the possibility and implications of temporal variations in atmospheric methane requires improved quantitative knowledge of methane sources and sinks. We report here methane flux measurements made over a 17-month period in the Great Dismal Swamp, Virginia. These flux measurements indicate that Great Dismal Swamp soils can act as both a source and sink for atmospheric methane. In a waterlogged condition, swamp soils are a net source of methane to the atmosphere with seasonal variations in emission rates from <0.001 to $0.02 \text{ g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$. During drought conditions, swamp soils consume atmospheric methane at rates of <0.001 to $0.005 \text{ g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$. While these results should not be extrapolated to all swamp soils, they illustrate the potential complexity of processes which regulate net flux of methane between wetland soils and the atmosphere.

Major natural sources of atmospheric methane involve anaerobic fermentation of organic material by microbial activity in wetland ecosystems and enteric fermentation in mammals⁷. While several laboratory and field investigations have identified variables correlated and coupled to the biogenesis of methane in soils and sediments^{7–13}, existing data are inadequate as a basis for quantitative estimation of global sources. Inadequate methodology for *in situ* quantitative measurements of methane flux at air–water and soil–water interfaces in wetland environments has been a primary limiting factor. Previous measurements have involved limited sampling of gas bubble flux through a water column or use of simple static chamber techniques. Flux measurements in static chambers have been criticized for producing serious modification of the atmosphere over the soil surface during long incubation periods. We have developed a new technique for gas flux measurements in wetland environments which combines a gas-filter-correlation (GFC) IR absorption analyser with improved sampling chambers that enclose a soil area (closed chambers) or chambers that are open to artificially induced air flow from outside (open chambers). This continuous sampling and analysis system has high precision ($\pm 0.01 \text{ p.p.m.}$) and fast response time (1 s) for measuring methane flux as low as $1 \times 10^{-4} \text{ g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in <20

min. The system is portable and floatable and is operated at remote locations using battery power. Flux measurements reported here were obtained with a 0.5 m^3 closed chamber. In the closed chamber mode, air continuously recirculates from the sampling chamber over the soil through the GFC and back to the chamber; any changes in methane concentration in the chamber are measured continuously. A circulation fan maintains an average air flow of 0.63 m s^{-1} in the chamber. Chamber temperature and humidity are maintained at ambient atmospheric values throughout a flux measurement. Calibration of the system is carried out each day in the field using certified methane standards. Details of instrument design, calibration and field testing are given elsewhere¹⁴.

A study of plant ecology, soils and nutrient cycling in the Great Dismal Swamp, Virginia (latitude $30^\circ 40' \text{ N}$, longitude $76^\circ 30' \text{ W}$) has provided an excellent opportunity to examine ecological and climatological factors in relation to temporal variations in methane flux. Extensive background data are available on the hydrology, ecology and land-use history of the Great Dismal Swamp^{15–19}. Measurements of water levels and soil and air temperatures were collected throughout our 17-month study period.

The Great Dismal Swamp is an 85,000-hectare, forested, peat bog and is one of the more extensive swamp forests still remaining in the USA²⁰. All of the methane flux measurements reported here were taken in a maple-gum habitat. Maple-gum (*Acer rubrum* and *Nyssa aquatica*) is the dominant vegetation in the swamp, with scattered stands of cypress (*Taxodium distichum*) and cedar (*Chamaecyparis thyoides*) as secondary components. Seasonal inundation of the maple-gum habitat varies in depth and frequency reflecting local rainfall. During the period of this study, we had an opportunity to study the

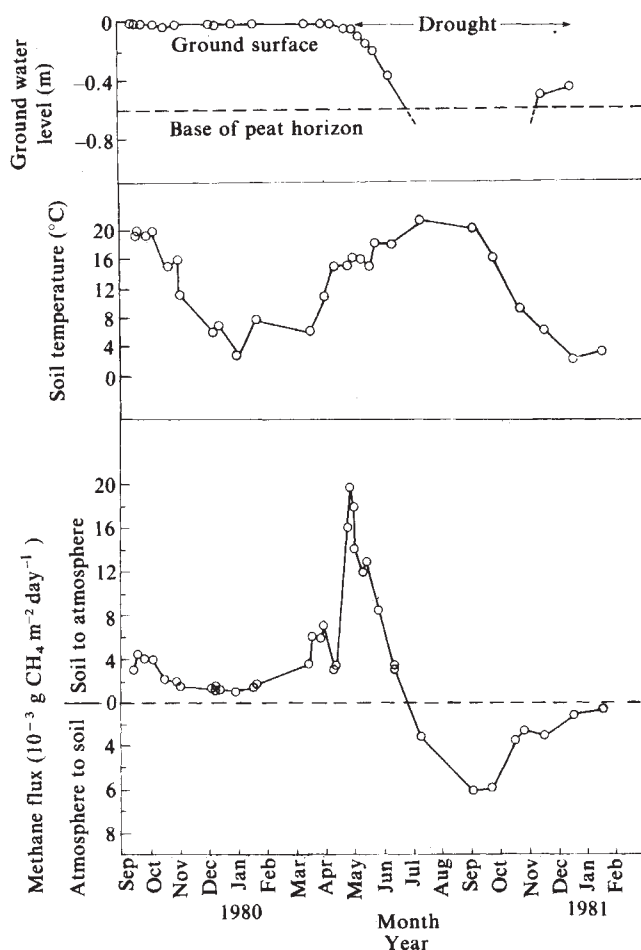


Fig. 1 Measured ground water levels, soil temperatures at 5 cm and methane flux in a Great Dismal Swamp peat soil between September 1979 and February 1981.

swamp in conditions ranging from totally flooded soils to dry soils resulting from drought conditions. All fluxes reported here are from soil or water surfaces without vegetation in the chamber.

Figure 1 summarizes temporal variations in water levels, soil temperature and methane flux for the Dismal Swamp maple-gum soil from September 1978 to February 1981. Two features of the methane flux variability are of particular interest. First, measured emissions of methane from waterlogged soils to the atmosphere ranged from a low of $1.3 \times 10^{-3} \text{ g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in December to a high of $19.7 \times 10^{-3} \text{ g CH}_4 \text{ m}^{-2} \text{ day}^{-1}$ in April. From September to December, methane emission rate decreased as soil temperature decreased. During March and April, methane emission rate increases as soil temperature increases. Note that for any specific soil temperature above 2°C , methane emissions in spring exceed fall rates.

Drawing analogy with studies in lakes, we hypothesize that microbial activity in the soil profile is stratified with methane oxidizing microorganisms living in the shallow aerobic surface layer and methane producing microorganisms living at depth in oxygen depleted habitat. Our measurements are net flux between the soil and atmosphere. During winter surface soils down to $\sim 2 \text{ cm}$ are at temperatures $< 1^\circ\text{C}$, while the remainder of the peat profile (2–60 cm) maintains temperatures of $2\text{--}4^\circ\text{C}$. Thus, the early spring maximum in methane flux to the atmosphere in April 1980, may represent a period when winter–spring production relative to oxidation rates reaches a maximum. Snow cover and frozen soils would both inhibit metabolic activities of methane oxidizing microorganisms in surface soils and reduce gas exchange across the soil–air interface, leading to an accumulation of methane in the soil during winter months. The pattern of methane evolution we observed in spring 1981 in the Great Dismal Swamp may be similar in origin to episodic gas evolution from Arctic tundra soils during spring thaw²¹.

Methane fluxes to the atmosphere from maple-gum peat soils in the Dismal Swamp are generally lower than flux rates measured for other wetland soils^{13,22–24}. Several factors including the enhancement of emissions by aquatic plants²⁵, differences in soil organic matter and nutrient content²⁶, and differences in measurement techniques could be contributing to the observed differences in flux rates. We do not claim that our measured flux rates in the Dismal Swamp are representative of other forested wetlands; rather we consider the primary importance of our data is in illustrating the potential complexity of processes which regulate long-term net flux of methane between wetland ecosystems and the atmosphere.

The second major feature in our study of temporal variations in methane flux is the reversal of the flux from July 1980 to February 1981. During this period, the peat soils in the maple-gum habitat of the Great Dismal Swamp were a sink for atmospheric methane. Methane removal from ambient air over the soil surface is associated with drought conditions. The water table dropped below the bottom of the surface peat, producing a relatively dry, porous soil. From October 1980 to February 1981, the methane flux from the atmosphere to the soil decreased as soil temperature decreased. While methane oxidation in certain lake and marine environments is well documented^{27–31}, we believe this is the first field measurement of a soil sink for atmospheric methane. Previous investigators concerned with the atmospheric methane budget have concluded that the soil is not a significant sink^{32,33}. While our results represent only one ecosystem, the question of dry peat soils as a sink for atmospheric methane should be considered an unresolved issue. Measurements over a normally dry soil under hardwood forest during this same time interval showed no detectable methane flux.

As an independent approach to the study of methane consumption in soil, we sampled gas from depths of 5, 12 and 33 cm in the peat soil. Small volume samples were drawn into evacuated flasks, followed by laboratory gas chromatographic analysis. Methane concentrations in soil gas decrease with depth from 1.6 p.p.m.v. (surface), 1.5 (5 cm), 1.4 (12 cm), to 1.0

p.p.m.v. (33 cm). Using these data to calculate methane flux from atmosphere to soil, assuming molecular diffusion is the primary transfer process, a value of $1.8 \times 10^{-3} \text{ g CH}_4 \text{ per m}^2 \text{ per day}$ is obtained. Our measured net flux at the soil–air interface using the GFC system on the same day was $2.6 \times 10^{-3} \text{ g CH}_4 \text{ per m}^2 \text{ per day}$. The slightly higher value for the directly measured flux is expected because factors such as the air flow over the soil surface can enhance transfer rates relative to molecular diffusion. The relatively close agreement between independent techniques adds further support to the concept of Dismal Swamp peat soils as a sink for atmospheric methane during dry conditions.

Our results raise questions concerning the generally accepted estimates of global methane emissions from wetlands and the assumption that soils are not a significant sink for atmospheric methane. Previous estimates of methane flux from major wetlands of the world have been based on a simple temperature–methane production relationship^{2,13}. Our results indicate that soil water content, temperature and other seasonal climatological factors are all potentially critical factors in determining whether a wetland soil is a net source or sink for atmospheric methane. Most of the major tropical freshwater wetlands of the world are subject to seasonal fluctuations in rainfall and water levels. The Florida Everglades, Okefenokee Swamp, Sudd and most rice fields are typically dry for at least several months of the year. In addition, extensive land reclamation projects are underway in the Everglades, Sudd and other wetlands which involve drainage and conversion of wetland soils to agriculture. These considerations indicate that a reassessment of the role of natural wetlands as a source of methane to the global atmosphere is necessary. Most importantly, our results illustrate the potential complexity of the problem of quantitatively determining long-term net flux of methane from a wetland ecosystem to the atmosphere.

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Evidence of colonial nesting and 'site fidelity' among ornithischian dinosaurs

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Recent discoveries in the late Cretaceous (Campanian) Two Medicine Formation of western Montana indicate that some dinosaur species, like some modern species of birds and crocodiles, nested in colonies. I report here evidence that members of one species returned to the same nesting area for many years. There are also indications that, after hatching, some species remained in their respective nests whereas others left the nest but remained in, or at least returned occasionally to, the nesting site. The area in which these discoveries were made is the Willow Creek Anticline of Teton County, where in 1978 a nest of fifteen, 1-m long hadrosaurs provided the first evidence of extended parental care among dinosaurs¹.

I have found in the same sediment horizon that produced the '1978 nest', a second nest of younger juvenile hadrosaurs (0.5 m long) and the weathered remnants of six unoccupied nests containing abundant eggshell remains (Fig. 1). All of the eight nests seem to have hosted conspecific hadrosaurs, of an as yet undetermined species, as the eggshell fragments from each site are structurally identical. Each nest appears to have been constructed as a circular or oval pit within a preconstructed mud mound (see ref. 1; Fig. 1). They were each found at an equal depth beneath a palaeosol, incorporated in a grey-brown mudstone interpreted as a floodplain deposit². Where erosion or cover has not destroyed or obscured parts of the nesting horizon, the nests are at least 7 m apart or approximately a distance equivalent to the length of an average adult hadrosaur from this area. There may, therefore, have been as many as 40 nests in the 10,000 m² area. The occurrence of eight nests along what appears to have been a single time horizon, within a relatively small area, suggests that these hadrosaurs were nesting in a colony.

With the exception of a single isolated juvenile in the nesting area, the remains of the young hadrosaurs were found in nests associated with eggshell. Although the skeletal elements of the young are relatively well ossified, even on the youngest individuals, there is no indication that they were precocial self-feeding individuals that would have ventured out of their respective nests without parental supervision, as suggested for

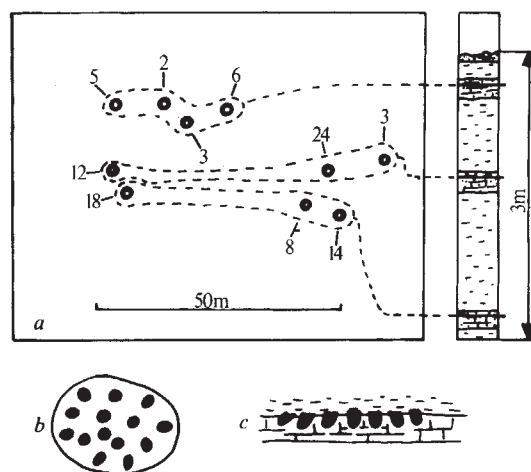


Fig. 2 a, Map and vertical section of the anticline showing the relationship of the egg clutches attributed to the hypsilophodont-like ornithopod. Values represent the number of eggs per nest, broken lines enclose clutches found on single horizons. b, Typical clutch arrangement viewed from above. c, Egg clutch viewed from the side showing how the lower portions of the eggs are located in siliceous carbonate sediment.

*Psittacosaurus*³. Early ossification may instead simply reflect rapid growth. As for the juveniles being too small for effective feeding by the adults, or possibly being crushed by an adult 'mis-step' (ref. 3), the fact that the crocodilians manage to overcome these difficulties⁴⁻⁶ implies that other archosaurs could also manage to do so. If by instinct or parental assistance, the young remained in their respective nests and the parent fed them from the nest perimeter, the young may have grown more rapidly, and there would have been less risk of infant mortality. Protection against predators would have been afforded by both nest confinement of the young and the closely-packed nests of the colony with the continued presence of many adults. As some adults foraged and gathered food for their young, the presence of other adults remaining in the nesting area may have thwarted potential predators, as is thought to be the case for many colonizing birds⁷. The presence of the two nests containing the remains of juveniles probably reflects this security in that they were not obviously preyed upon. Their deaths may have been due to starvation after the death of their parents.

A second nesting site (Fig. 2) on Willow Creek Anticline which has yielded several juvenile ornithopods, closely allied to the Hypsilophodontidae, offers evidence not only of colonial nesting but also of 'site fidelity' (multi-year use of nesting site) and the possibility of bird-like creches. The 10 nests attributed to these ornithopods contain the hatched remains of up to 24 eggs per clutch. Clutches that do not appear to have suffered erosional damage are ~1 m in diameter, and were found along at least three different horizons, incorporated in brown, organic-rich, siliceous carbonates with mudstone inclusions². These are interpreted as products of soil formation where partially dried sediments were repeatedly disrupted and mixed in the process of nest construction and daily traffic². Although the exposed site of these nests is being excavated, the number of clutches so far found along these three horizons suggests colonial nesting for this species. The fact that they occur at different levels indicates that the members of the species returned to the site for many years. The elongate, ellipsoid eggs attributed to these ornithopods were meticulously laid with their smaller ends 'planted' upright to oblique, partially into the siliceous carbonate sediments (Fig. 2). On hatching, the young left through the top portion of the eggs, leaving the lower portions intact within the sediment. The fact that the lower portions were preserved rather than crushed and broken by trampling of the young, together with the fact that juvenile remains were not found in the nests, suggests that the young of this species did not remain in the nests long after hatching. It is possible, however, that

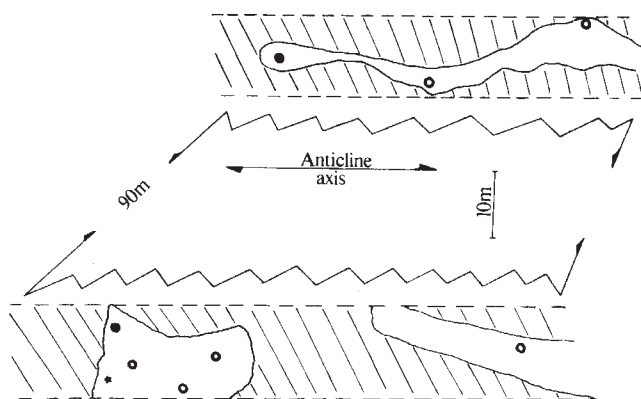


Fig. 1 Relationship of hadrosaur nests straddling Willow Creek Anticline. ●, Nests containing juveniles; ○, nests with eggshell concentrations and the asterisk indicates the location of the isolated juvenile. Broken lines represent the limit of the nest-bearing horizon. Cross-hatched areas are covered or missing.

the juveniles from different nests assembled into bird-like crèches, as the remains of at least 12 small skeletons of varying sizes, have been found on the nesting horizons. Adults of this species appear to have been ~2.5–3 m long. The juvenile remains represent individuals ~0.5–1.5 m long, indicating that the young of this species either remained in the colony or returned to the site frequently, either of which may have been a result of parental care.

Considering the variety of nesting habits and social behaviours exhibited by the different crocodilian species or particularly by Recent birds, it is expected that the morphologically and ecologically diverse dinosaurs would also have exhibited a variety of behaviours.

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Lucy's limbs: skeletal allometry and locomotion in *Australopithecus afarensis*

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Precise information about the bodily proportions of early hominids is crucial for accurate functional and phylogenetic interpretations of early human evolution^{1–6}. The partial skeleton of *Australopithecus afarensis* (AL 288-1; 'Lucy'^{7,8}) recovered in 1974 from the Hadar area of Ethiopia⁹ permits the first direct assessment of body size, limb proportions and skeletal allometry in ancestral hominids that pre-date 3 Myr. Using allometric relationships for limb lengths in non-human catarrhine primates (as a whole and for African apes alone) as empirical base lines for comparison, I show here that the limb proportions of *A. afarensis* are clearly unique among hominoids. The data indicate that *A. afarensis* had already attained forelimb proportions similar to those of modern humans but possessed hindlimbs that were relatively much shorter; hence the 'intermediate' humerofemoral index of AL 288-1 (85.1) compared with *Homo sapiens* and great apes^{9,10}. It follows that relative and absolute elongation of the hindlimbs represents one of the major evolutionary changes in later human evolution. The bodily proportions of Lucy are not incompatible with some form of bipedal locomotion, but kinematic identity and functional equivalence with the bipedal gait of modern humans seem highly improbable. Reduced relative stride length in AL 288-1 probably implies both greater relative energy cost and relatively lower peak velocities of bipedal locomotion in *A. afarensis*.

Speculation about the limb proportions of the earliest hominids has long preoccupied palaeoanthropologists and functional anatomists^{1–6,11–15}, largely due to the obvious importance of such information for realistic reconstructions of locomotor behaviour and probable phylogenetic pathways. Due to the lack of associated, complete limb bones from both fore- and hindlimbs of *Australopithecus*, the earliest unequivocal hominid, comparisons of bodily proportions with modern *H. sapiens* have been necessarily tentative and inconclusive. Some

authors suspect that australopithecines possessed proportions virtually identical to those of modern humans, perhaps for the entire postcranial skeleton^{16,17} or just for the hindlimb^{2,18}. Others have proposed that the forelimbs were relatively long^{7,12,15} and/or the hindlimbs relatively short^{4,6,12,14}. The recently prepared, partial skeleton of *A. afarensis* (AL 288-1; 'Lucy') includes complete elements of forelimb (humerus) and hindlimb (femur), and therefore provides the first opportunity to test previous inferences about limb proportions and skeletal allometry in ancient hominids. As Walker³ notes, it is only when such data are available that "biomechanical analyses of the postcranial parts of *Australopithecus* will become more convincing than they are at present".

Based on a skeletal sample of 454 adult, non-human catarrhine primates (Old World monkeys, lesser apes and great apes) and wildshot body weights, I have established allometric relationships for humerus length and femur length in catarrhines as a whole (a size spread from *Miopithecus talapoin* to *Gorilla gorilla*) and in African apes alone (from female *Pan paniscus* to male *G. gorilla*)¹⁹. The sample includes 164 cercopithecines, 77 colobines, 116 hylobatid apes and 97 pongids. Body weights are taken from museum records and the literature. These scale relationships serve in this analysis as comparative, empirical baselines from which vertical deviations (d_{yx}) can be identified in AL 288-1, a sample of modern *H. sapiens* and a small female bonobo chimpanzee. For prediction and d_{yx} calculation, model I regression of $\log_{10}$ -transformed data is regarded as more appropriate^{19–21} than model II methods (although principal axis solutions yield identical conclusions). Least-squares regression of $\log_{10}$ (humerus length) and $\log$ (femur length) on $\log_{10}$ (body weight)^{1/3} yields the following predictions for catarrhines as a whole:

$$\text{Humerus: } \log_{10} Y = 1.048 \log_{10} X + 0.862 \quad (r = 0.88)$$

$$\text{Femur: } \log_{10} Y = 0.828 \log_{10} X + 1.192 \quad (r = 0.94)$$

For African apes only, the predictive equations are:

$$\text{Humerus: } \log_{10} Y = 0.815 \log_{10} X + 1.229 \quad (r = 0.99)$$

$$\text{Femur: } \log_{10} Y = 0.437 \log_{10} X + 1.806 \quad (r = 0.94)$$

Following Smith^{17,21}, percentage deviations ('prediction errors') for humerus length and femur length were computed from each equation as

$$\frac{\text{Observed value} - \text{predicted value}}{\text{Predicted value}} \times 100$$

for AL 288-1, a female Mbuti pygmy *H. sapiens* (MMC-18)²², male and female samples of caucasian *H. sapiens* and a female bonobo *P. paniscus* (T-29060). Body weight of Lucy has been estimated by her discoverers at 60 lb (27.3 kgf)⁷. A conservative 25.0–30.0 kgf range has been used in this analysis, consistent with independent estimates derived from both linear²³ and multiple (C. O. Lovejoy, personal communication) regression. Body weight of the Mbuti pygmy was calculated elsewhere²⁴, and the caucasian male and female values represent actual weights at death (Table 1). At 27 kgf the wildshot female bonobo falls at the lower limit of adult body size in *P. paniscus*. It is included in this analysis specifically because it is a pongid skeleton that is similar in size to both AL 288-1 and MMC-18.

Percentage deviations (Table 1) disclose a close similarity between AL 288-1 and both *H. sapiens* groups in relative humerus length. The humerus is slightly shorter than predicted in all the hominids, whereas that of the female bonobo is longer than expected. In contrast, prediction errors for femur length indicate a great disparity between modern humans on the one hand and Lucy and T-29060 on the other. With the exception of AL 288-1 relative to African apes, femoral deviations are positive in all cases, but those of the modern human groups are much greater than Lucy's. The same results obtain even if an estimate of 50 lb (22.7 kgf) is used for Lucy's weight. Inter-group allometric projections (Table 2) reinforce these observations. An AL 288-1 to caucasian projection results in near isometry ($k = 1.10$) for humerus length but extreme positive

Table 1 Deviations (d_{yx}) from allometric relationships in extant catarrhine primates

	BW (kgf)	HL (mm)	FL (mm)	% Deviation from catarrhine line		% Deviation from African ape line	
				HL	FL	HL	FL
<i>A. afarensis</i> (AL 288-1) ♀	25.0–30.0	239	281	–4.5 to –10.4	+4.8 to +10.2	–9.9 to –14.3	–2.1 to +0.1
<i>H. sapiens</i> Mbuti pygmy ♀ (MMC-18)	27.3–30.9	256	349	–0.8 to –5.0	+29.1 to +33.6	–5.8 to –8.9	+21.1 to +23.3
<i>H. sapiens</i> Caucasian ♂ ($n=9$)	68.3 (±5.2)	336 (±17)	469 (±27)	–5.6	+39.4	–3.6	+44.9
Caucasian ♀ ($n=9$)	55.0 (±5.5)	308 (±11)	433 (±18)	–6.6	+36.6	–6.3	+38.1
<i>P. paniscus</i> (T-29060) ♀	27.0	289	291	+12.5	+11.6	+6.7	+2.9

Percentage deviation calculated as (observed value – predicted value)/(predicted value) × 100 (see refs 17, 21). Catarrhine allometric equations are model I regression estimates for a size run from *M. talapoin* to *G. gorilla*; African ape allometry equations are model I regression estimates for a size run from female *P. paniscus* to male *G. gorilla*. Model II regressions (such as principal axis) yield similar conclusions, but are statistically inappropriate for calculations of vertical residuals (d_{yx}). The body weight of AL 288-1 has been estimated at 60 lb (27.3 kgf)⁷; a conservative range of 25–30 kgf is used here. The body weight values for MMC-18 are from ref. 17. Body weights of the caucasian skeletal sample are from the autopsy records of the Cleveland Museum of Natural History; only non-pathological (neither obese nor malnourished) individuals were measured. The body weight of T-29060 is taken from the field notes at the Musée Royal de l'Afrique Centrale in Tervuren. Lengths of humerus and femur of AL 288-1 are taken from reconstructions made by C. O. Lovejoy at the Cleveland Museum of Natural History. BW, body weight; HL, humerus length; FL, femur length.

allometry ($k=1.72$) for femur length. A pygmy to caucasian projection, by contrast, reveals near isometry for both skeletal elements. Bonobo to caucasian scaling requires extreme relative reduction of the forelimb ($k=0.43$) coupled with extreme positive allometry of the femur ($k=1.58$), comparable with the Lucy–human vector. Because AL 288-1, MMC-18 and T-29060 had approximately equal body weight, a direct comparison of their limb dimensions produces the same conclusion in a slightly different manner. Lucy possesses the shortest humerus of the three, but the Mbuti pygmy humerus is only 7% longer; that of the bonobo is 21% longer. Lucy also possesses the shortest femur, but that of the bonobo is <4% longer; the femur of MMC-18 is almost 25% longer than Lucy's. As the crural indices (tibia/femur × 100) of African apes and humans are very similar^{19,25}, a short femur necessarily implies that the entire hindlimb was comparably short. All analyses lead to the same conclusion—that forelimb proportions of *A. afarensis* (as represented by AL 288-1) were already comparable with those of modern humans, but that the hindlimb of this early hominid was relatively short and proportionately similar to that of a small pongid. Earlier suspicions^{4,6,12,14} of relatively short hindlimbs in *Australopithecus* are confirmed by these findings, but predictions^{4,7,12,15} of relatively long forelimbs are not substantiated (at least in these gracile australopithecines). These results also account for the intermediate humerofemoral index (85.1) of AL 288-1 compared with *H. sapiens* and pongids^{9,10}.

Dramatic hindlimb elongation (absolutely and relatively) emerges as one of the major evolutionary changes from *A. afarensis* to modern humans. This finding contrasts with the recent assertion²³ that natural selection would be unlikely to favour relative hindlimb lengthening in a short-limbed ancestral hominid. A major proportional difference between Lucy and a comparably sized ape is the shorter forelimbs of the former. Analogy to the locomotor behaviour of great apes and basic biomechanics of climbing^{19,26} suggest that the climbing capability of *A. afarensis* was probably diminished by such interlimb proportions. Unfortunately, we do not know whether Lucy's forelimb proportions are primitive or derived for African hominoids. Nevertheless, we do know that to derive *A. afarensis* from a relatively small-bodied hominoid with limb proportions similar to *P. paniscus* would entail significant forelimb reduction but little change in hindlimb dimensions^{6,14}. Because we do not know whether the immediate ancestor of *A. afarensis* had proportions comparable with bonobos, this evolutionary scenario cannot be tested. Given Lucy's pongid-like hindlimb proportions, reconstructions of stature in *A. afarensis* derived solely from metric trends evinced by modern humans²⁷ also become less convincing.

Diagnostic details of the knee joint and bony pelvis of *A. afarensis* are compelling indicators of a bipedal adaptation^{7,28–30}, and fossilized footprints^{27,31} provide dramatic evidence of bipedal locomotion in *A. afarensis*. However, the relatively short hindlimb of Lucy implies substantial kinematic differences in bipedal gait from the modern condition. Both relative and absolute stride length were necessarily reduced in AL 288-1. The range of flexion and extension at the hip would have had to have been much greater in Lucy to match the stride of, say, MMC-18, but nothing in the anatomy of the hip joint would indicate an exaggerated degree of potential excursion³². These findings probably also account for the relatively short stride reconstructed for the makers of the Laetoli footprints³³ without having to resort to hypothetical 'strolling' behaviour.

Relative elongation of the hindlimb in later hominids would permit increasing velocity of gait at only a slight increase in energy cost because increased speed can be achieved by increased stride length rather than by increases in cadence (step frequency)^{34–37}. Selection to reduce the energy cost of bipedal locomotion³⁸ would therefore favour hindlimb elongation, although relatively long extremities are probably also advantageous for enhanced performance (such as greater maximum terrestrial velocity). Thus the hindlimb proportions of *A. afarensis* and other 'primitive' details of the foot, ankle and hip^{32,39} cannot be construed as evidence of a fully modern adaptation to bipedalism^{31,33,40}.

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Table 2 Intergroup allometry coefficients ($y = \beta x^k$)

Pairwise groups	Exponent	
	Humerus	Femur
<i>A. afarensis</i> → Caucasian (AL 288-1) (means)	1.10	1.72
Mbuti pygmy → Caucasian (MMC-18) (means)	0.93	1.04
<i>P. paniscus</i> → Caucasian (T-29060) (means)	0.43	1.58

'Exponent' is estimated by the least-squares (model I) regression of $\log_{10}$ (limb length) on $\log_{10}$ (body weight)^{1/3}. The weight of AL 288-1 used here is 60 lb (27.3 kgf); that of MMC-18 is the midpoint value of 29.1 kgf. An exponential value of 1.0 indicates isometry or geometric similarity.

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Unchanged thermoregulatory set-point in the obese mouse

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The genetically obese (*ob/ob*) mouse has been extensively studied as a model of early-onset obesity. These mice are characterized by a wide variety of endocrine and metabolic defects including hyperglycaemia, hyperinsulinaemia with insulin resistance, hyperlipidaemia and hyperphagia^{1,2}. The marked obesity of this mutant cannot be attributed to over-eating because it remains fat even when fed the same amount of food as its lean littermate^{3,4}. The earliest indicators of the *ob/ob* genotype, low metabolic rate and low body temperature, are detectable 7–10 days after birth^{5,6}. Hypothermia might, therefore, be a prime contributor to the development of obesity because a greater percentage of the diet will be diverted to fat rather than 'wasted' for thermogenesis^{7–9}. As the obese mouse exhibits hypothermia over a wide range of ambient temperatures, it has been suggested that their set-point for body temperature regulation is lower than that of the lean mouse¹⁰. If this is the case the obese mouse should maintain a subnormal body temperature when required to work for exogenous heat in a cold environment. We now report that the set-point is unchanged in the obese mouse because it behaviourally defends a body temperature equivalent to that of the lean mouse during cold exposure.

Four pairs each of obese male and female and lean male and female C57BL/6J mice, 12–16 weeks old at the beginning of the experiments, were housed from birth in a colony room at 23 °C. The test apparatus consisted of a 24 × 10 × 18 cm cage with a 3 × 5 cm Plexiglas lever mounted at one end. The naive mice were trained to press the lever until stable rates of heat intake were observed when the cage was placed in a freezer at -8 ± 2 °C. Each response activated two 250-W IR lamps mounted outside the cage and focused on the area around the lever. The total power dissipated by the lamps was controlled at 300 W, giving a radiant flux density of 180 mW cm⁻² at the lever. The lamps remained on as long as the lever was depressed. The time that the heat lamps were on and the total number of responses were recorded on counters in an adjoining room.

Body temperature was measured before and after each test session using a Yellow Springs telethermometer and 402 rectal probe (2.5 cm insertion). After training and one additional practice session, each mouse was tested for 2 h every 3 or 4 days until four tests were completed.

Obese mice placed in the -8 °C test environment did not maintain their body temperature at the initial value of 34.8 °C (Table 1). Instead, they raised their temperature by almost 2 °C to a level comparable with that of the lean littermates. Although normothermic, their rates of heat intake were lower than those of lean mice. Lean females (25.1 g) obtained the most heat (26.1 s of heat per min), while obese males (50.7 g) and females (48.4 g) obtained the least. These relationships between body weight and heat intake suggest that the size of the animal significantly affects its exogenous heat requirement. This supposition is supported by the very high correlation coefficient ($r = -0.93$) between body weight and heat intake. The higher rates of heat intake in the lean mice might also reflect a greater heat loss between responses because they were far more active than the obese mice. Invariably they sat on the lever in order to activate it and then explored the cage until the next response. In contrast, the obese pressed with their forepaws but remained huddled in front of the lever when the lamps were off. This behaviour would minimize heat loss and also maximize heat intake because some radiant energy is emitted from the filaments of the lamps as they cool. The obese mice also pressed very briefly as if testing the lever during the interval between longer responses, and thereby they emitted twice as many lever presses as the lean mice (Table 1).

In another 2 h session we determined the latency to normothermia in obese and lean mice. Rectal temperatures were measured 5, 15, 30, 60, 90 and 120 min after the start of a test period. Both lean and obese mice had substantially elevated body temperatures within 5 min of the beginning of the session (Fig. 1). Peak temperatures were seen 10 min later and the values remained near 37 °C for the last hour of the test. When the intensity of the heat lamps was varied, all animals responded by increasing heat intake when the wattage was decreased and by reducing it when the wattage was increased. Thus, both

Table 1 Pre- and post-test body temperatures and heat intake during tests at -8 °C

Group	n	Body temperature (°C)		Heat intake	
		Before	After	Responses min ⁻¹	Heat min ⁻¹
Lean	8	36.5 ± 0.17	36.9 ± 0.15	2.9 ± 0.46	24.8 ± 0.57
Obese	8	34.8 ± 0.19*	36.6 ± 0.15	6.1 ± 0.64*	20.6 ± 0.33*

Values are the mean ± s.e.m.

* Significant ($P < 0.01$) difference by *t*-test between lean and obese groups.

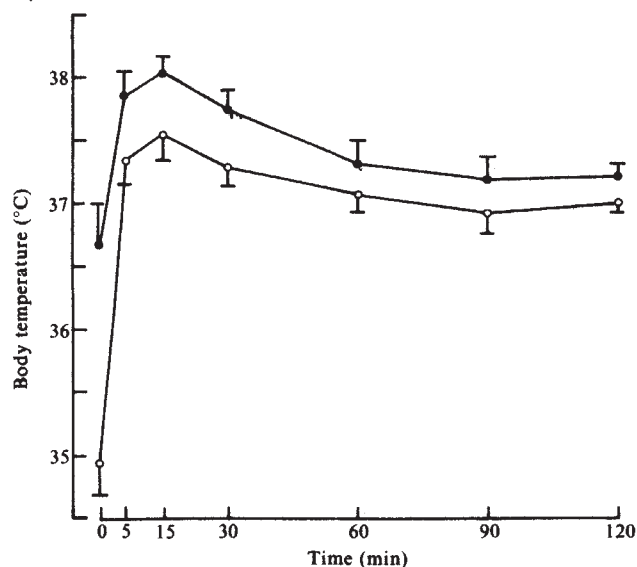


Fig. 1 Mean body temperatures ($\pm$ s.e.m.) of eight lean (●) and eight obese (○) mice during the behavioural heat intake tests at -8°C .

phenotypes responded appropriately and equally to variations in reward magnitude.

We also attempted to determine whether the trained mice would work for heat in a neutral ambient temperature. The mice were placed in the defrosted freezer in standard test conditions, except that the initial ambient temperature was $23 \pm 1^{\circ}\text{C}$ and could be raised if the mice worked for heat. In separate tests of 1 and 2 h, all mice raised the ambient temperature by working for heat. The obese mice raised the chamber temperature to 26.5°C during the 1 h test and to 27.8°C during the 2 h test. Their average body temperature at the end of the sessions was 36.0°C , or 1°C higher than their initial temperature. The lean mice raised ambient temperature to 29.0°C during the 1 h test and to 30.9°C during the 2 h test. Average body temperature increased from 36.9 to 37.4°C . The lean mice obtained an average of 2.8 s of heat per min during the 1 h test and 2.2 s min^{-1} during the 2 h test, whereas obese mice obtained 1.2 and 0.84 s min^{-1} respectively. Thus, the obese mice raised their body temperature more but did not attain the same body temperature as the lean mice, nor did they obtain as much heat, or raise ambient temperature to the same level as the lean mice. We therefore conclude that the preferred ambient temperature of obese mice is 2.5 – 3.0°C lower than that of lean mice. This effect may be due to the difficulty of increasing heat dissipation in a warm environment because of adiposity. It is also possible that the obese mouse, known for its low activity¹¹, may be unwilling to expend the effort necessary to raise ambient temperature above a minimally acceptable level. Other experiments are necessary to verify these alternatives.

The factors responsible for the hypothermia-hypometabolism of *ob/ob* mice remain to be defined. Our data indicate that hypothermia is not the result of a diminished requirement for heat or of an impaired sensitivity to ambient temperatures because the obese mouse behaviourally defends the same body temperature in a cold environment as the lean mouse. The diversion of dietary energy to fat rather than to heat must involve a defective or inappropriate heat production mechanism, possibly related to abnormal thyroid hormone production^{12–15}, or depressed hepatic¹⁶ or brown fat thermogenesis^{17–20}.

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The rigidity of fish and patterns of lateral line stimulation

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Various functions have been attributed to the lateral line organs of fish (and amphibia), including those of detecting touch and sound (both near- and far-field) and flow past a swimming fish^{1–4}. As the lives of fish and the structures of their lateral lines vary greatly, lateral line function almost certainly varies between species and is probably not simple even for one animal. It is, however, generally agreed that lateral line neuromasts are excited by liquid within the canal moving relative to the canal walls^{4–6}. For the sprat, such movements are proportional to local differences in motion between the fish and the surrounding seawater⁷ and a similar situation must exist for other fish. Here we describe the motions of fish and seawater at various positions around vibrating sources. We show that fish are rigid longitudinally, and thus local differential movements between fish and seawater occur. Predictions based on these results suggest that when a fish is close to a source of vibration, for example, to a neighbouring fish, the amplitudes, signs and patterns of stimulation along the lateral line system change in a striking way with the position of the fish relative to the source.

Freshly killed fish (*Sprattus sprattus*, *Clupea harengus*, *Ammodytes lanceolatus* and *Trachurus trachurus*) were suspended by fine threads in a seawater tank at various distances and bearings from a vibrating source and at various angles with

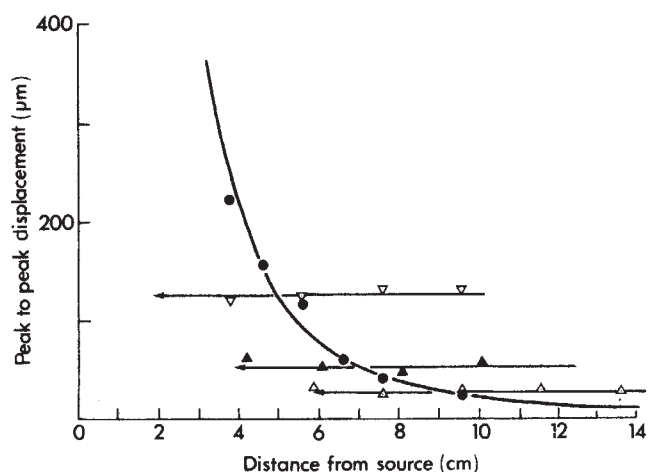


Fig. 1 The longitudinal rigidity of the sprat. In this case the fish is lying along the axis of the bellows source (12 Hz), with its head towards the source. Ordinate, peak-to-peak displacement; abscissa, distance from source. Three positions of the fish are shown; arrows indicate the snout. Triangles indicate observed fish displacement. ●, Observed water displacement; the curve shown is the best fit of a power function.

respect to a radius to the source. *S. sprattus* and *C. harengus* have lateral lines on the head only, whereas *A. lanceolatus* and *T. trachurus* have them on the head and body. The sound sources, a pulsating bellows and a vibrating sphere, were driven sinusoidally between 12 and 40 Hz. The resulting displacements of fish and water were observed with a dissecting microscope by measuring the lengths of bright lines made by the movements of small light-scattering particles in the skin of the fish and in the water. The corresponding pressures were measured with a ceramic transducer.

In any given situation, the longitudinal components of displacement of the fish (for all the species) were constant at all points, which demonstrates that these fish behave as rigid bodies longitudinally. For the small displacements used, the fish could bend freely in directions perpendicular to the vertebral column so that the amplitude of motion across the fish could differ greatly at different points along its length.

Figure 1 shows, for example, water and fish movements for a sprat with its long axis in the axis of vibration of the bellows source, at various distances from the source. The movement of the fish could generally be predicted reasonably well by assuming it to be a rigid cylinder driven by the pressure difference between its ends. While always behaving as a rigid body longitudinally, this simple method of calculation proved inadequate when the distance from the vibrator was small compared with the length of the fish.

Further studies were done using a vibrating sphere, a source more representative of a beating tail. Measurements of the amplitudes and directions of displacements and of pressures around the vibrating sphere in a seawater tank agreed well with the predictions of the near field terms of the equations given by Harris⁸. The pressures associated with such vibratory sources were much larger than we had wrongly thought⁶. We also measured movements of fish suspended at various positions with respect to the source. The pressures at the head and base of the tail were calculated and the expected motion of the fish derived from the pressure difference and the fish's length and density. Table 1 compares calculated and experimental values for the longitudinal displacements of a fish for several positions with respect to the source. As the agreement was good, it seemed reasonable to calculate displacements of the fish for other positions and also the local differences in movement between the fish and the seawater (the effective stimuli to the lateral line). Figure 2 shows such differences and how they change along the length of the fish for a source whose frequency and amplitude of motion and size are similar to those of the tail of a swimming sprat. Typically, there were one or two positions along the length of the fish at which there was no such difference. For these positions there would be no local stimulation of the lateral line. The polarity of the lateral line stimuli differ for different parts of a fish. This is probably of importance because some sensory cells in each neuromast are excited by displacements of the cupula towards the fish's head and others by displacements towards the tail. Figure 2 also gives values of pressure, longitudinal acceleration and transverse acceleration calculated for the position of the ear; in clupeids the ear contains the pressure⁷ and acceleration detectors.

Table 1 Movement of fish in the field of a vibrating sphere (16 Hz)

Fish no.	r (m)	θ (deg)	ψ (deg)	ξ_{exp} (μm)	ξ_{calc} (μm)
1	0.058	1	181	90	110
2	0.087	68	132	45	31
3	0.085	66	0	52	52
4	0.104	26	85	7	7
			Mean	48.5	50

r , θ , Polar coordinates of the snout from the centre of the source (zero angle in the axis of motion). ψ , Angle of the fish to the axis of motion of the vibrating sphere. ξ , Peak-to-peak displacement of the fish along its longitudinal axis: exp, experimental; calc, calculated.

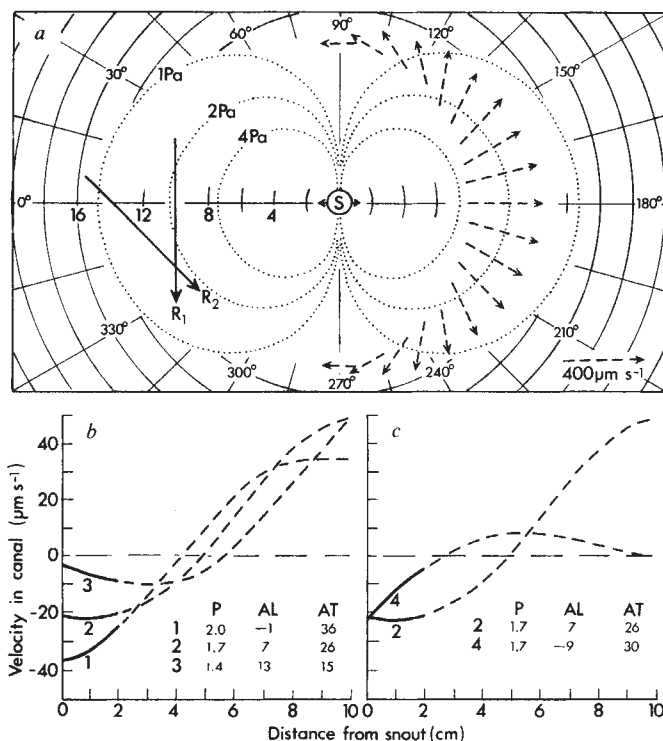


Fig. 2 *a*, Sound field in relation to the fish positions considered at the bottom of the figure. S, source, a sphere of radius 0.75 cm vibrating at 16 Hz with peak-to-peak displacements of 1.0 cm. R, 'receiving' fish, a 10 cm-long sprat, about one fish length from S. The scale in cm is shown along the 0°–180° axis. The fish are indicated by solid black lines (R_1 and R_2) with arrowheads at the snout. Dotted lines are isobars; broken lines with arrowheads show the directions and amplitudes of particle velocities in the medium. *b*, *c*, The abscissae show the distance from the snout along the length of the fish. Ordinates, velocities of the liquid in the lateral line canal (relative to the walls of the canal). The tabulated values are all peak-to-peak; P, pressure (Pa); AL, longitudinal acceleration of fish (mm s^{-2}); AT, transverse acceleration. The signs indicate the polarities during one half-cycle. The solid line shows the effects for the full extent of the sprat lateral line. Solid plus broken line indicates the relative effects for a lateral line extending to the tail. *b*, Effect of R passing S along the line of R_1 in *a* (mimicking a fish changing station in a shoal): (1) fish 2 cm behind position labelled R_1 ; (2) fish in position R_1 ; (3) fish 2 cm ahead of position R_1 . *c*, Effect of R changing its angle by 45°: (2) fish in position R_1 ; (4) fish in position R_2 .

For the sprat we know the relationships between direction, frequency and amplitude of seawater movements outside the fish and movements within the lateral line canals⁷. Making an informed guess at the sensory thresholds from the results of various experiments (refs 7, 9 and some unpublished results), we estimate that the sprat lateral line system excited in this way could detect a neighbouring fish in a shoal at distances of up to a few fish lengths.

These experiments relate to situations that are simpler than the natural ones. They do show, however, for both a vibrating and a pulsating source, that the stimulation of lateral line sense organs (by a neighbouring source) falls more rapidly with distance and changes more dramatically with position than might be expected. The stimulations of the lateral line, otolith¹⁰ and pressure-sensitive sense organs all change in different ways on moving around a vibrating (Fig. 2) or pulsating source. In this array of sense organs, those of the lateral line, in which the pattern of stimulus changes greatly with position, are well suited to having a major sensory role in such activities as the schooling of sprat and saithe¹¹ or, in the case of rays, searching for prey.

When the distance of a source is great relative to the length of a fish (this can happen well within the near field), the motion of the water at the head of the fish will be very similar to that at the tail. The differences in motion between fish and water

will thus be very small, therefore the stimulation of the lateral line by the mechanism discussed here will be of little consequence. This means that lateral line systems should be disturbed little by most of the background noises in the sea^{4,6,12}.

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Place navigation impaired in rats with hippocampal lesions

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Electrophysiological studies have shown that single cells in the hippocampus respond during spatial learning and exploration^{1–4}, some firing only when animals enter specific and restricted areas of a familiar environment. Deficits in spatial learning and memory are found after lesions of the hippocampus and its extrinsic fibre connections^{5,6} following damage to the medial septal nucleus which successfully disrupts the hippocampal theta rhythm⁷, and in senescent rats which also show a correlated reduction in synaptic enhancement on the perforant path input to the hippocampus⁸. We now report, using a novel behavioural procedure requiring search for a hidden goal, that, in addition to a spatial discrimination impairment, total hippocampal lesions also cause a profound and lasting place-navigational impairment that can be dissociated from correlated motor, motivational and reinforcement aspects of the procedure.

If rats are placed in a large circular pool of opaque water, they will quickly learn to escape by finding and climbing on to a small platform hidden beneath the water surface, provided it remains in a fixed location over a series of trials⁹. They cannot learn to find it when its position varies randomly from trial to trial. Although they can never see, hear or smell the platform, rats require only a few trials in order to learn to swim directly towards it, using the shortest route, even from a novel starting place. That is, the rats learn not only to recognize the vicinity of the safe place when they reach it, but also to swim towards it from a distance despite the absence of cues from the platform itself. The deleterious effects of cue-response separation apparent in visual discrimination^{10,11} do not, in this case, prevent extremely rapid learning. By comparing the performance of normal and brain-lesioned animals in these conditions with that shown when a fixed but visible platform was used, we have examined the role of the hippocampus in simple navigation.

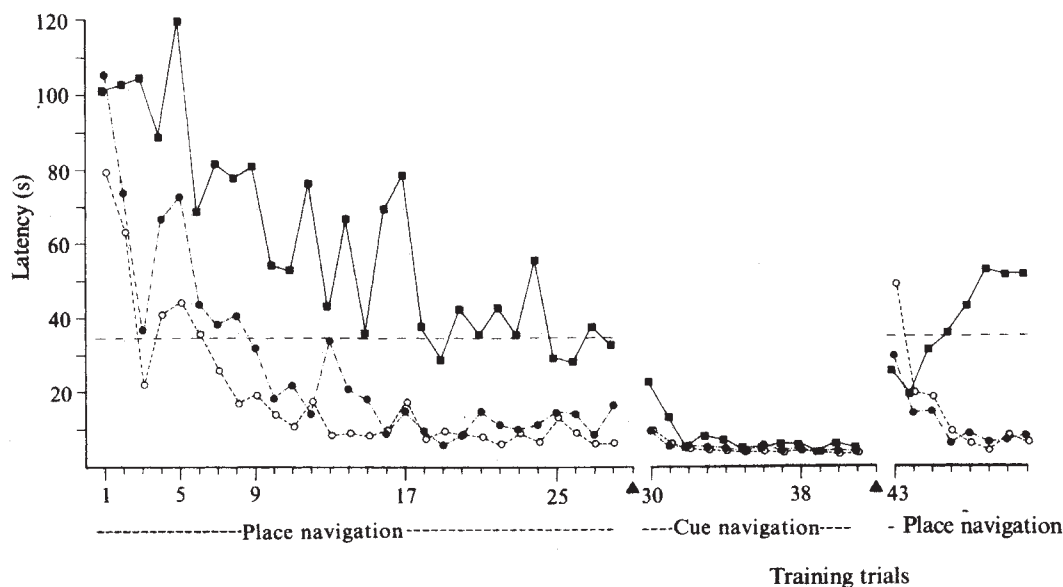
Female Lister rats ($n = 31$) were subjected to the following procedures: total hippocampal lesions ($n = 10$), superficial cortical lesions ($n = 13$), sham surgery ($n = 4$) or no surgery ($n = 4$). The rats were placed, under pentobarbitone anaesthesia, into a special adjustable head-holder¹². Animals in the hippocampal lesion group had holes drilled in their skulls, and a small amount of neocortex overlying the hippocampus, and the entire dorsal and ventral hippocampus were removed by aspiration. Operated control animals had comparable lesions in the neocortex but showed no hippocampal damage. Sham-operated control animals had burr holes drilled in their skulls but suffered no brain damage. On completion of the behavioural procedures, conventional histological techniques (40 μ m, gelatine-embedded sections stained with cresyl violet and solochrome cyanide) were used to verify the lesions. All rats of the hippocampal-lesion group were found to have total or near total destruction of the dorsal and ventral hippocampus, with minimal damage to adjacent structures (comparable lesions are reported in ref. 13). Analysis of the behavioural data showed no differences between the sham-operated and unoperated control groups, which were therefore combined, giving final group sizes of 10 (hippocampal), 13 (cortical) and 8 (control).

On day 1, the rats were placed in a pool of water (1.32 m diameter, 53 l at $26 \pm 1^\circ\text{C}$) and allowed to swim freely for 1 min with no opportunity for escape. On day 2, a platform was hidden in one of four locations in the middle of each cardinal quadrant (SW, NW, NE and SE), 0.33 m from the side walls. Different locations were used for different rats. The platform, made of clear perspex, was hidden by adding 2.3 l of milk to the water and arranging for its top surface, 8 cm in diameter, to be 1 cm below the water level. A second platform, 2 cm taller and protruding visibly out of the water, was used at a later stage of training; its top surface was indented such that it contained within its circumference a 1 cm layer of water. Thus, the reinforcement afforded by escape on to the two platforms was equated. The rat's task throughout the training procedures, which continued for 8 days, was to find and escape on to the platforms. Only one platform was used at a given stage of training, and it was always in a fixed position in the pool on a given day. Thus the two tasks, which we shall call place-navigation and cue-navigation, respectively, involved the same motor movements (swimming), motivation and reinforcement (escape from water), but differed specifically and uniquely with respect to whether or not the rat was required to learn the platform's position in relation to the varied distal room cues.

All rats swam effectively using the characteristic adult swimming posture¹⁴. The times taken to escape from the water during the three successive phases of the experiment are shown in Fig. 1. The normal and cortical-lesion groups learned to escape rapidly from the water with stable terminal acquisition latencies of < 8 s. The hippocampal-lesion group showed a highly significant impairment in the place-navigation task (trials 1–28) when the hidden platform was used. However, this impairment declined dramatically and disappeared when the visible platform was used (trials 30–41), this platform having been placed diagonally opposite to the earlier training location (that is, NW for a rat trained previously to find the hidden platform at SE). The place-navigation impairment reappeared when training was continued with the hidden platform (trials 43–50) even though it remained in the same position that the visible platform had occupied in the preceding phase of training.

Detailed analysis of the behavioural performance of each group and the results of two transfer tests provide new insights into the nature and magnitude of the deficit after hippocampal lesions. First, the hippocampal-lesion animals did improve during training but never escaped faster than normal animals searching for a hidden platform that was moved around randomly from place to place on successive trials (see Fig. 1, horizontal broken line, taken from ref. 9). Second, analysis of the paths taken by all rats on trial 28, transcribed from videotape recordings, showed that the hippocampal-lesion animals took longer and more circuitous routes to find the hidden platform

Fig. 1 Mean latency of escape (s) for the 50 trials of the experiment. ■, Hippocampal lesion; ●, cortical lesion; ○, control. The trial number of the first of each daily set of trials is shown on the abscissa. The two transfer tests are indicated by solid triangles. To avoid problems of heterogeneity of variance, the successive phases of the experiment were analysed separately. The horizontal broken line (trials 1–28 and 43–50) at 34.5 s corresponds to the best performance shown by a group of normal rats trained to search in 20 trials for a hidden platform that was moved randomly from one place to another over successive trials (data taken from ref. 9). Place navigation (trials 1–28): unweighted means (unequal *n*) analysis of variance revealed significant effects of group ($F = 23.7$, d.f. = 2/28; $P < 0.0001$), trials ($F = 17.8$, d.f. = 23/667; $P < 0.0001$) and lesion $\times$ trials ($F = 1.5$, d.f. = 54/756; $P < 0.02$). Subsequent orthogonal comparisons showed that the deficit was restricted to the hippocampal-lesion rats (hippocampal versus cortical + control, $P < 0.0001$; cortical versus control, $P > 0.10$). Cue navigation (trials 30–41): terminal escape latencies (trial 41) were 5.0, 3.3 and 2.8 s for the hippocampal-lesion, cortical-lesion and control groups respectively, corresponding to declines relative to trial 28 of 45.9, 7.9 and 3.7 s. Analysis of variance of all 12 trials revealed a small residual impairment in the hippocampal-lesion groups ($F = 5.4$, d.f. = 2/28; $P < 0.02$). Return to place navigation (trials 43–50): analysis of variance showed a highly significant effect of groups ($F = 12.2$, d.f. = 2/28; $P < 0.0002$) and a lesion $\times$ trials interaction ($F = 3.6$, d.f. = 14/196; $P < 0.0001$). The apparent gradual impairment of performance in the hippocampal-lesion group was caused by a slowing of swimming speed over trials as the core temperature of the rats fell slightly (from 37 °C to ~35 °C).



(Fig. 2). The directional heading of the hippocampal-lesion rats when they set off from their starting position on trial 28 was no more likely to be towards the platform than in any other possible direction. These results imply that hippocampal-lesion rats can learn some sort of escape strategy (for example, that escape is possible) but are substantially poorer at learning where the hidden platform is located and, unlike normal and cortical-lesion animals, will never learn to swim towards it from a distance.

The magnitude of this place-navigational deficit was assessed in two separate transfer tests conducted on trials 29 and 42, immediately after the four daily trials of days 6 and 8. For transfer test A, the hidden platform was first removed from the apparatus, then the rats were placed in the pool for 60 s with no opportunity for escape, and their movements observed. The results were striking. Control and cortical-lesion rats swam to and persistently across the former platform location whereas the hippocampal-lesion rats did not. The hippocampal-lesion rats did not merely swim around the side walls. To demonstrate this, annuli were marked on the video screen indicating the exact surface area and former positions of the platforms in each of the four cardinal quadrants. The total number of annuli which an individual rat passed through during the 60-s test was 7.6, 6.8 and 8.6 for the hippocampal-lesion, cortical-lesion and control groups, respectively ($F < 1$). The groups were distinguished by which annuli they passed through: an individual hippocampal-lesion rat was no more likely to pass through the annulus marking the platform position used during training than one in any other quadrant (Fig. 3a). We observed no tendency on the part of the hippocampal-lesion rats to remain in the vicinity of the training annulus once they had eventually reached it (compare with ref. 7). Thus the deficit produced by hippocampal lesions was total. Furthermore, with respect to the lack of spatial bias revealed in the annulus measure, the deficit was apparent in all 10 rats of the experimental group.

Our interpretation of these findings is that, whatever their other effects^{15–19}, hippocampal lesions do cause a profound and lasting place-navigational impairment. It could be argued, however, that while matched for motor requirements, motivation and reinforcement, the place- and cue-navigational tasks are not matched for task complexity. Perhaps hippocampal-lesion animals perform poorly on the spatial task because it is

complex (albeit a task learned by normal animals in less than 10 trials), and perform better on the visible platform task because it is easier, rather than because the spatial component is then redundant. If this is the basis of the dissociation of effects in the two tasks, then at least some spatial bias should be shown by some of the hippocampal-lesion animals in a transfer test conducted after training on the ostensibly easier visible platform task. Transfer test B, conducted immediately after trial 41 on day 8, examined this possibility. In trial 41 itself, there was no significant difference in the latency, path-length or directionality of escape behaviour across groups ($F_s < 1$), all animals escaping rapidly by means of short, direct paths to

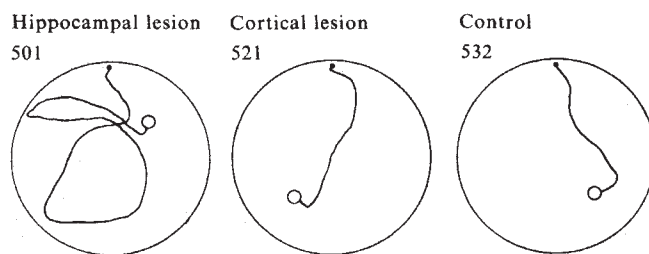


Fig. 2 The actual path of the median rat (defined in terms of path length) in each group on trial 28 just before the first transfer test. The rats were observed using a video camera placed above the pool. One experimenter (P.G.) sat concealed in one corner of the room and monitored the rat's movement on a VTR recorder. The second experimenter (R.M.) removed each rat from its home cage in an adjacent room and placed it in the pool. The pool was open to the room which included a door, a window, and brightly and darkly lit walls. The paths taken by the rats in escaping were transcribed from the videotape and measured. Path lengths: the hippocampal-lesion rats took 4.66 ± 0.86 m to reach the platform, whereas the cortical-lesion and control rats took 2.35 ± 0.98 and 1.20 ± 0.34 m, respectively. Analysis of variance showed that these path lengths differed significantly ($F = 4.23$, d.f. = 2/28; $P < 0.025$). Subsequent orthogonal comparisons showed that the hippocampal-lesion group took significantly longer paths than both the cortical-lesion and control groups ($P < 0.001$), which in turn did not differ significantly from each other ($P > 0.10$). Directionality: the accuracy of the approach to the platform was analysed as follows. We measured the angle subtending a tangent to the rat's path at a point 0.5 m from its starting position, and a line intersecting this point and the centre of the platform. This angle was $83 \pm 18^\circ$ from the correct direction for the hippocampal-lesion group, whereas for the cortical-lesion and control groups, the angle was $34 \pm 13^\circ$ and $38 \pm 17^\circ$, respectively (Kruskal-Wallis, $H = 5.87$, d.f. = 2; $P < 0.025$, one-tailed).

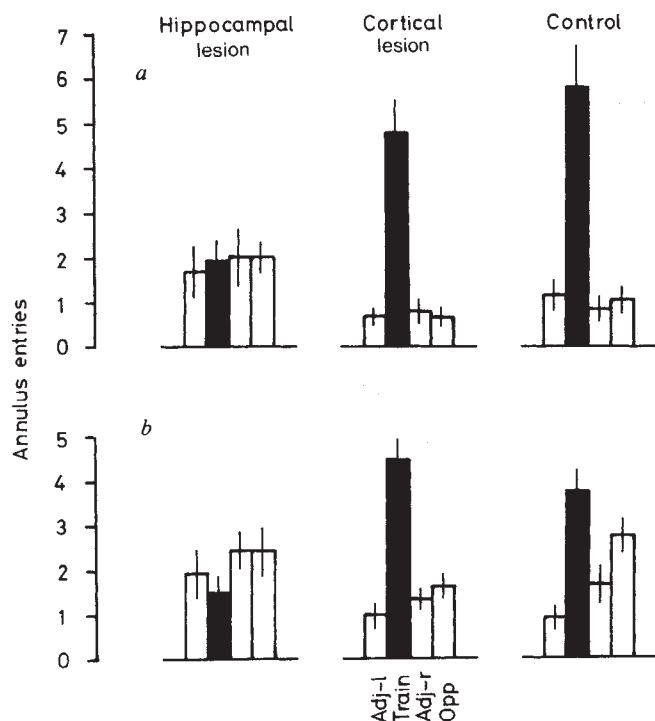


Fig. 3 Mean crossings of each of the annuli (± 1 s.e.) marking the former platform positions during *a*, transfer test A (after place-navigation training) and *b* transfer test B (after cue-navigation training). The data have been categorized for each animal into crossings of the training location (Train), and of the annulus in the adjacent quadrant to the left (Adj-l; viewed from above), the adjacent right (Adj-r), and opposite quadrant (Opp). Note that the hippocampal-lesion rats were no more likely to pass through the annulus marking the training position than any other, in both transfer tests. Analyses of variance showed a highly significant groups $\times$ position effect in both transfer test A ($F = 10.1$, d.f. = 6/84; $P < 0.0001$) and transfer test B ($F = 9.5$, d.f. = 6/84; $P < 0.0001$).

the visible platform. However, in the transfer test conducted 30 s later, only the control and cortical lesion groups searched in the vicinity of the now absent but previously visible platform (Fig. 3*b*). All 10 animals in the hippocampal-lesion group showed no spatial bias. Thus even if the improvement by the hippocampal-lesion group in the visible platform phase of training was due to the simplicity of the task, this improvement was not accompanied by any spatial learning.

The procedures used here provide a new approach to analysing the brain mechanisms of spatial localization. The results show that hippocampal lesions cause a profound and lasting impairment in place-navigation and question that aspect of the working-memory hypothesis¹⁷ which asserts that spatial reference memory is unaffected by septo-hippocampal damage. Reference memory has been defined as those aspects of a learning procedure in which learned information may be used in every trial of training rather than for just a single trial. The present procedure using a fixed platform position for 28 trials, followed by different fixed position for 20 further trials, is certainly a reference-memory procedure. In the absence of separate measures of working memory in this experiment, we cannot comment further on the adequacy of that hypothesis. However, we suspect that claims about the integrity of spatial reference memory after more restricted fimbria-fornix lesions and extensive preoperative training²⁰ may provide a misleading picture of normal hippocampal function.

The present results show that normal rats can navigate in an appropriate direction towards a hidden object; they do not merely recognize a place when they reach it. Whether this type of learning involves or is different from conventional associative learning deserves further scrutiny. But given that place units detected so far in the hippocampus¹⁻⁴ respond only with respect to places in which the rat is presently situated as opposed to places to which it intends to go, these results pose a challenge

for electrophysiologists attempting to explain the neural mechanisms by which the hippocampus processes spatial information.

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Evidence for dendritic competition in the developing retina

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At present little is known of the rules regulating dendritic morphology. Several studies have demonstrated that the shape of the dendritic tree depends on its afferent supply^{1,2}. The ganglion cells of the retina provide a particularly useful cell type for the study of neurone development as they develop independently of afferents from other brain regions. If the ganglion cells alone are destroyed in a small patch of the developing retina, it is possible to examine how the absence of neighbouring neurones of the same type influences the development of the ganglion cells around the depleted area. The development of the normal laminar pattern of the retina is not disturbed by the loss of these cells³. We show here that the dendrites of ganglion cells around the depleted area are preferentially directed towards this region. The orientation of ganglion cell dendrites is strongly influenced by neighbouring cells and we suggest that during normal development, dendrites compete for their afferents.

Experiments were performed on 11 hooded Lister rats. On the day of birth, the rats were anaesthetized by hypothermia and a small lesion was made in the temporal retina of one eye using a fine 28-gauge needle passed through the sclera approximately half-way between the optic disk and the limbus. After 2-3 months, the animal were anaesthetized with an intraperitoneal injection of 3.0 ml per kg of chlor-nembutal (2.1 g of chloral hydrate + 0.5 g of sodium pentobarbital in 50 ml of 0.9% saline). A series of six injections of 0.15-0.25 μ l of horseradish peroxidase (HRP; Boehringer) (50% w/v in 2% dimethyl sulphoxide) were made stereotactically into the optic tract, using a 1- μ l Hamilton syringe. The animals were killed painlessly after 24 h, perfused with 0.9% saline and the eyes removed. The retinæ were prepared as whole mounts⁴, and reacted in a modified Hanker-Yates solution⁵. After washing in 0.1 M phosphate buffer (pH 7.2) for several hours, each retina was transferred to 50 ml solution of 0.1 M sodium

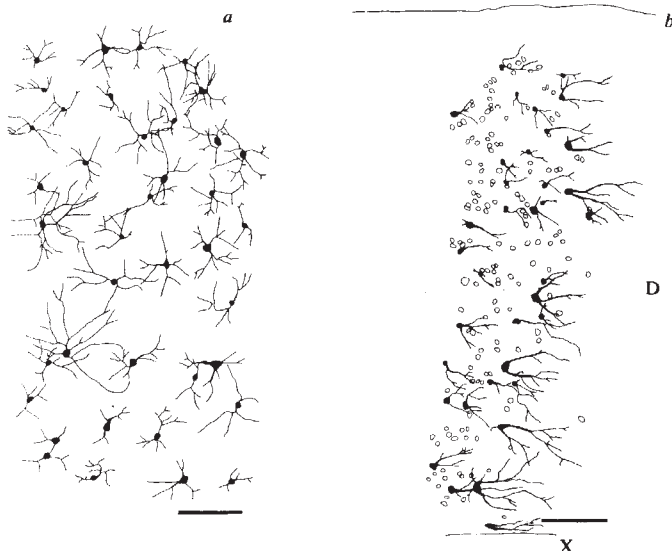


Fig. 1 *a*, Drawing of the cell bodies and proximal dendrites of ganglion cells filled with HRP. The HRP reaction product did not fill the entire dendritic tree. The cells are located ~3 mm nasal to the optic disk in a retina with a lesion located temporal to the optic nerve head. They were drawn to give an approximately evenly spaced distribution; other labelled neurones are not shown. *b*, Drawing of the cells along the edge of a region devoid of ganglion cells (D). The site of entry of the needle is marked by X; the line at the top of the figure indicates the periphery of the retina. The cell bodies of some faintly filled cells are drawn in outline to indicate the border of the region devoid of ganglion cells. The dendrites and cell bodies of well-filled cells are drawn completely. Reproduced from an original drawing made at $\times 400$. Scale bar, 250 μm .

cadodolate buffer (pH 5.1) containing (mg) ammonium nickel sulphate (200), cobalt chloride (300), *p*-phenylenediamine (25) and catechol (50). They were incubated in this solution for 15 min, then washed in phosphate buffer for 3–5 min and transferred to a fresh solution of 50 ml of 0.1 M sodium cacodylate buffer (pH 5.1), containing *p*-phenylenediamine (25 mg), catechol (50 mg) and 1 drop of 30% hydrogen peroxide. After incubation for 15 min, the retinæ were again washed in phosphate buffer and mounted on a gelatinized slide. The retinæ from several normal animals (that is, having no lesions) injected with HRP were treated in the same way as those from the experimental animals and served as controls. The retinæ from three of the experimental animals were fixed in 10% formol-saline, prepared as whole mounts and stained with cresyl violet. In addition, retinæ from animals in which one optic nerve had been sectioned at birth, were stained with a Nissl stain either as whole mounts or in vertical sections⁶.

In the retinæ of the normal rats, many ganglion cells were filled sufficiently with HRP to reveal their proximal dendrites. It proved difficult to follow the dendrites for a long distance before they became lost in the dense network of the inner plexiform layer, except for a few cells where a large part of the dendritic tree was intensely stained and resembled a Golgi-stained neurone. As has been previously reported for the rat and other animal species^{7,8}, most ganglion cells have circular or elliptical dendritic fields but some have dendrites predominantly on one side of the cell body. Within any given patch of retina, however, such cells showed no consistent asymmetry (see Fig. 1). The only exceptions are the outermost rim of the retina, along which ganglion cell dendrites tend to be oriented towards the ora serrata, and the region of the major blood vessels where the cell bodies have been displaced laterally away from their underlying afferents.

In the experimental retinæ, the lesion site was readily identified because the ganglion cell layer peripheral to it was almost totally lacking in ganglion cells. The needle cut the axons

of the cells peripheral to the lesion in a wedge-shaped segment of the retina. These ganglion cells had degenerated, in agreement with previous studies which showed that neonatal ganglion cells do not survive transection of their axons⁶. At the lesion site there was some disruption of the retinal layers but in the peripheral region lacking ganglion cells, the laminar organization was normal for except for some shrinkage of the inner plexiform layer. The thickness of the retinal layers was measured in animals in which one optic nerve had been sectioned at birth, and which were kept alive for up to 11 months. The inner plexiform layer had shrunk by as much as 50% whereas the other layers showed only 3% shrinkage compared with normal. The area of the retinæ with the optic nerve sectioned was similar to the normal retinæ and the loss of the ganglion cells did not disrupt the formation of the normal lamina pattern^{3,6}. We therefore conclude that despite the loss of ganglion cells, the bipolar and amacrine cells which constitute the afferent supply of the depleted area, remained largely intact.

In the HRP-stained retinæ, it was apparent that the dendrites of the ganglion cells adjoining the lesion site had their dendrites directed almost exclusively towards the region lacking ganglion cells. This was true irrespective of cell soma size, and included the occasional displaced ganglion cell in the inner nuclear layer (Figs 1*b*, 2). This effect on the orientation of the dendrites can be seen all around the depleted area, irrespective of the shape of this area (Fig. 3) and is therefore probably not a consequence of any simple mechanical distortion. Ganglion cells located at least 400 μm from the edge of the depleted area were found to have distorted dendrites.

The results show that the orientation of developing ganglion cell dendrites is strongly influenced by neighbouring cells. The dendritic fields of developing ganglion cells are predominantly radial⁹, but in these experiments we observed cells having almost all their dendrites on one side of the cell body. We do not yet know the sequence of events which results in this dramatic reorganization of the dendritic geometry; the dendrites may be attracted to the depleted region and/or repelled by their neighbours on the other side. We suggest that during normal development, ganglion cell dendrites compete with each other for their afferent supply from bipolar and amacrine cells.

There is considerable evidence that the axon terminals of ganglion cells and other neurones compete for their postsynaptic territory during development^{10,11}. In the rodent retina, many ganglion cells degenerate in the first few days postnatal^{12,13}. The degeneration of ipsilaterally projecting cells can be partially prevented by removing one eye at birth, thus reducing the competition between the developing optic axon terminals^{12,14}. We have recently shown¹⁵ that after transection of one optic tract at birth, the number of cells which project ipsilaterally to the brain from the eye contralateral to the tract section is

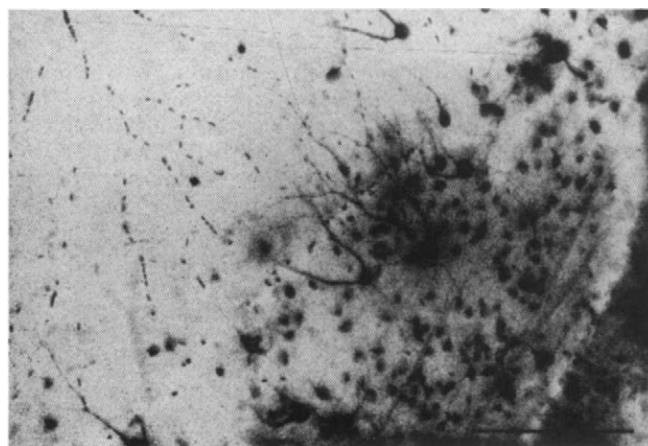


Fig. 2 Photomicrograph showing some of the ganglion cells with their dendrites directed towards a region lacking ganglion cells. Scale bar, 250 μm .

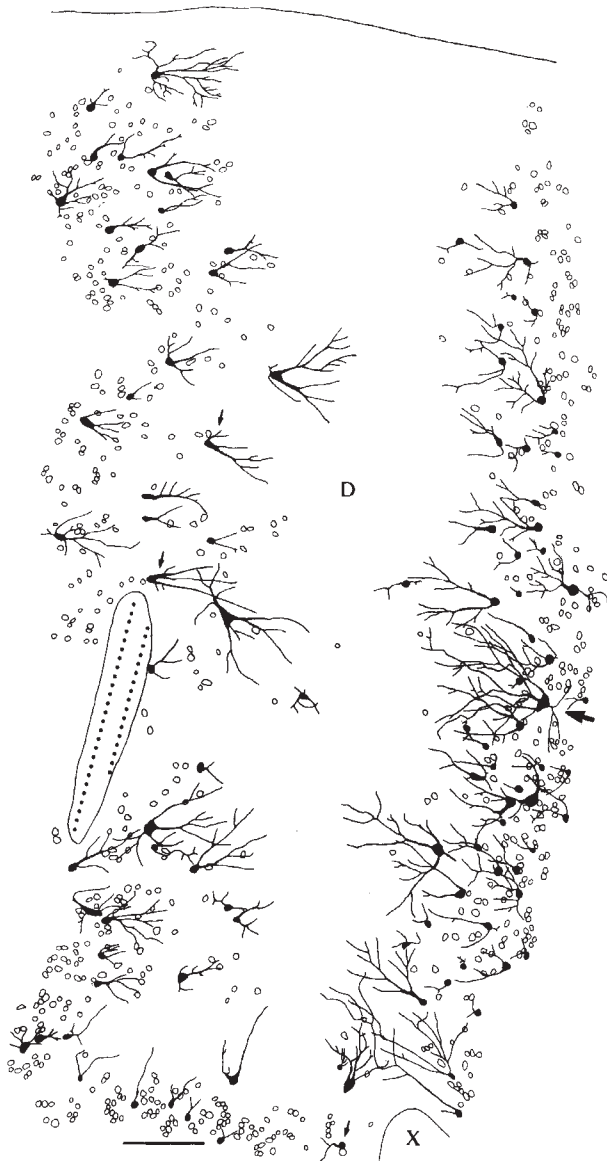


Fig. 3 Drawing of a small rectangular region of retina lacking ganglion cells, showing surrounding HRP-filled neurones. Conventions as for Fig. 1b. Stippling represents an area of post-mortem damage. The small arrows indicate displaced ganglion cells. The large arrow indicates the large cell illustrated in the centre of Fig. 2. Scale bar, 250 μ m.

increased. This is accompanied by an increase in the area occupied by the ipsilateral terminal field. This increase in the ipsilateral projection occurs despite the fact that the crossed projection from the other eye remains intact and that the expanded projection is found away from regions receiving commissural fibres. Thus the effect is not simply due to a change in competition at the axon terminals. We suggested that this abnormal population of ganglion cells survived in the retina as a result of reduced dendritic competition since the optic tract damage induced retrograde degeneration of almost all the contralaterally projecting ganglion cells. The results presented here provide evidence for such an interaction between adjacent developing ganglion cell dendrites.

It has been shown that different retinal cell types are distributed in regular mosaics across the retina¹⁶ and that the dendritic trees of a particular cell class are arranged so that every part of the retina is covered by the dendrites of that cell type¹⁷, presumably as a result of local interactions^{16,18}. If the population of ganglion cells in the developing retina is largely in excess of that found in the adult^{12,13}, a regular mosaic can

be generated by dendritic competition regulating cell death. Ganglion cells which fail to make a sufficient number of contacts with their afferents would die in much the same way as neurones which fail to make the appropriate postsynaptic contacts also die^{12,14}. The shape of the dendritic field of a given cell will result from the orientation of the dendrites changing in accord with available afferent supply. It remains to be seen whether other aspects of dendritic geometry, such as the length of the dendrites and frequency of branching, can also be influenced by the density of neighbouring cells and whether these interactions are limited within each given class of ganglion cell or level of branching in the inner plexiform layer.

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Incorporation of cytoplasmic vesicles into apical membrane of mammalian urinary bladder epithelium

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The mammalian urinary bladder is a distensible organ which undergoes a series of slow fillings and rapid emptyings (micturition), during a 24 h period. Recent evidence suggests that the bladder epithelium undergoes a three-phase, structural change during the expansion stage of this cycle^{1,2}. First, macroscopic multicellular folds are smoothed then microscopic folds are stretched out, resulting in a flattening of the urine-facing (apical) membrane. The last and most speculative phase is an incorporation or fusion of cytoplasmic vesicles with the apical membrane, resulting in an alteration of the cell shape from cuboidal to squamous. Such fusion of cytoplasmic vesicles is interesting not only from the point of view of the bladder as a storage reservoir for the discharge of the kidneys, but also from the perspective of hormonal regulation of the ion reabsorptive processes in this epithelium^{1,3}. Recently, the determination of the electrical properties of the apical membrane of mammalian urinary bladder has revealed that aldosterone (a Na⁺-conserving hormone) increases both Na⁺ conductance and selectivity⁴. The origin of such highly Na⁺ selective channels is unknown. Here we report that mammalian urinary bladder epithelium accommodates stretching by incorporating into the apical membrane vesicles from a cytoplasmic pool, which are withdrawn to effect recovery from stretch. The vesicle membrane contains Na⁺-selective channels—this has implications for the regulation of membrane transport.

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Rabbit urinary bladder epithelium was dissected, stripped of underlying muscular coats and mounted in modified Ussing chambers (2 cm² nominal area) designed to eliminate edge damage as previously described¹. Both sides of the epithelium were bathed at all times with identical solutions. Open circuit potential (T_T), trans-epithelial resistance (R_T) and short-circuit current (I_{sc} , a direct measure of Na⁺ transport) were continuously monitored and stored on a laboratory computer.

Capacitance, which is directly proportional to real apical membrane area (1 $\mu\text{F} \sim 1 \text{ cm}^2$ of membrane)¹ was monitored according to the method of Lewis and de Moura (unpublished), in which the epithelial capacitance is independent of cellular resistances and alterations in cellular or junctional resistances but is directly proportional to apical membrane area. The normal bathing solution had the following composition (mM): 111.2 NaCl, 25 NaHCO₃, 5.8 KCl, 2 CaCl₂, 1.2 MgSO₄, 1.2 K₂HPO₄ and 11.1 glucose, gassed with 95% O₂-5% CO₂ buffered at pH 7.4 at 37°C.

To investigate the phenomenon of intracellular vesicle translocation and apical membrane fusion, we performed three sets of experiments. In the first experiments, we stretched the epithelium and monitored the capacitance as a function of the stretch. Two approaches can be used to determine vesicle fusion: the first is to correlate tissue dry weight per cm² of chamber opening with the membrane area per cm² of chamber opening (that is, the capacitance). As the epithelium is stretched, both membrane area per cm² (capacitance, C_T) and dry weight per cm² will decrease linearly. In the absence of membrane insertion, both C_T ($\mu\text{F cm}^{-2}$) and dry weight (mg cm^{-2}) will reach minimum values. If at this point cytoplasmic stores of membrane (having a capacitance of 1 $\mu\text{F cm}^{-2}$) are inserted into the apical membrane, then C_T will remain constant while dry weight will continue to decrease. Preliminary experiments by Lewis and Diamond¹ demonstrated a biphasic response of capacitance to dry weight. At low levels of stretch both C_T and dry weight (per unit chamber area) decreased in a linear manner. With increased stretch, C_T changed only by 15% (to a minimum of 1.1 $\mu\text{F cm}^{-2}$) while dry weight decreased by 230%. These data strongly support the proposition that during stretch the urinary bladder epithelial cells change from a goblet shape (having a flat apical membrane) to an oblate spheroid (disk shape) by incorporating cytoplasmic membrane into the apical surface. The capacitance of this added membrane, estimated from the asymptotic capacitance value, is $\sim 1 \mu\text{F cm}^{-2}$. Does the cell have to alter its volume during stretch? It is easily demonstrated that given a cell of fixed basolateral area, the apical surface area can be increased by 245% at constant cell volume. Alternatively, one can decrease the weight by 245% at constant apical area.

The second and more satisfactory approach for determining vesicle fusion uses hydrostatic pressure. After mounting the preparation in a chamber modified so that it has no mesh backing, the epithelium is bowed towards the serosal solution by applying an excess of solution to the mucosal solution. At a constant weight (that is, constant mass of exposed tissue) and smooth apical surface area, this epithelium can only increase its capacitance by increasing its surface area. In three separate preparations, excess solution was added to the mucosal chamber. The capacitance (that is, apical membrane area) increased by $18 \pm 3\%$ ($\pm \text{s.e.m.}$, $n = 6$). After eliminating this hydrostatic pressure gradient, the capacitance returned to within $6 \pm 2\%$ ($\pm \text{s.e.m.}$, $n = 6$) of the starting value. This initial experiment indicated that mammalian urinary bladder accommodates stretch by incorporating vesicles from a cytoplasmic pool into the apical membrane and that recovery from stretch results in a removal of membrane back into the cytoplasm. Both the increase and decrease in capacitance occurred within at least 5 min after applying or removing the gradient.

In the second set of experiments, the epithelial cells were induced to swell by replacing the normal mammalian Ringer's solution with one of half osmotic strength. Changes in membrane area were again measured using capacitance as a criterion for area increase^{1,5}, where an increase in capacitance indicates a proportional increase in apical area. Figure 1a illustrates that on application of a hypo-osmotic solution there was no measurable capacitance change until ~ 15 min later. Apical area then rapidly increased for the next 45 min, reaching a stable value 74% greater than the control level. Returning the solutions to isosmotic resulted in a rapid return of the apical area to near control values. The decrease in capacitance was well defined

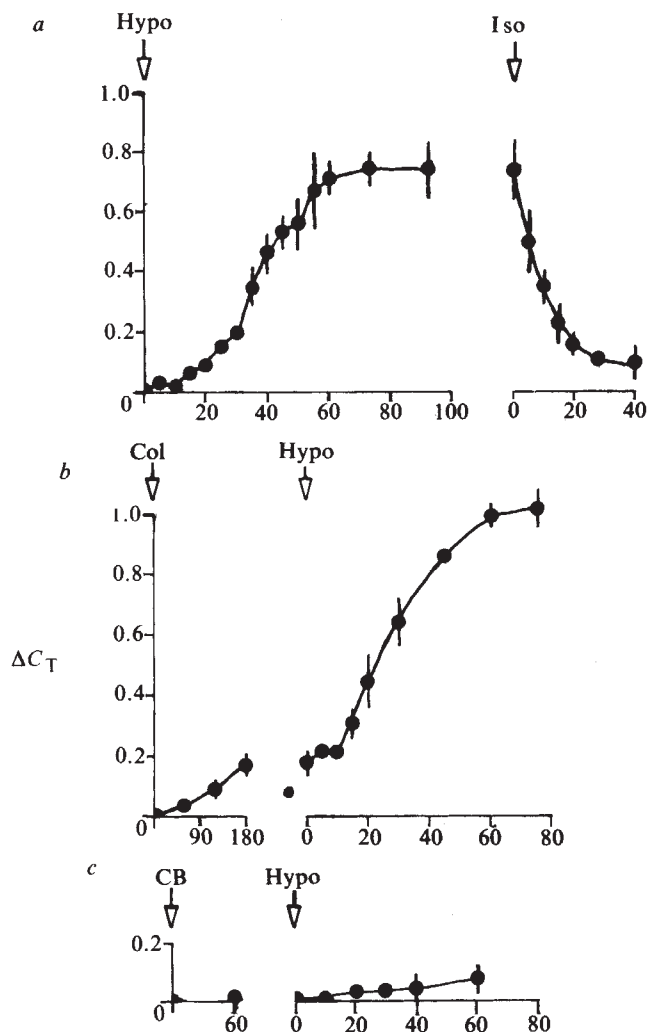


Fig. 1 Increase in normalized capacitance (ΔC_T) as a function of time after both mucosal and serosal solutions were replaced with a solution of half osmolarity. Capacitance was determined by passing a square current pulse across the epithelium, digitizing the voltage response and then fitting the voltage response by the sum of two exponentials. The best fit capacitance values (C_1 and C_2) are related to the actual apical (C_a) and basolateral (C_{bl}) capacitances by

$$C_T = \frac{C_1 C_2}{C_1 + C_2} = \frac{C_a C_{bl}}{C_a + C_{bl}}$$

As $C_a:C_{bl}$ is 1:5 (ref. 5), then C_T is an underestimate of C_a . If a change in C_T is produced by an increase in only C_{bl} , then C_T can change by a maximum of only 20% (that is, C_{bl} becomes infinite). The values shown in the figure indicate that the increase in C_T was almost completely accounted for by an increase in C_a , not C_{bl} . Capacitance was normalized by subtracting the measured capacitance from the zero time capacitance and dividing this difference by the zero time capacitance. Vertical bars represent the s.e.m. **a**, Time course of change in C_T after changing the bathing solutions (at $t = 0$) to half osmolarity (Hypo) and back to isosmotic solutions (Iso). Actual capacitance was $1.14 \pm 0.07 \mu\text{F cm}^{-2}$ ($n = 8$) at $t = 0$ and $1.97 \pm 0.13 \mu\text{F cm}^{-2}$ at $t = 92$ min. **b**, Pretreatment of bladder with colchicine (Col) for 3 h caused, in normal solutions, a $17 \pm 4\%$ increase in C_T . Exposure to half-osmolarity solutions (Hypo) resulted in a rapid increase in capacitance. A return to control solution caused a rapid decrease in C_T to control values (not shown). Actual capacitance was $1.3 \pm 0.13 \mu\text{F cm}^{-2}$ ($\pm \text{s.d.}$, $n = 3$) at $t = 0$ (before colchicine treatment). **c**, Bladders were pretreated with cytochalasin B (35 μM ; CB) for 90 min and then exposed to half-osmolarity solutions (Hypo). Note that there was no change in C_T .

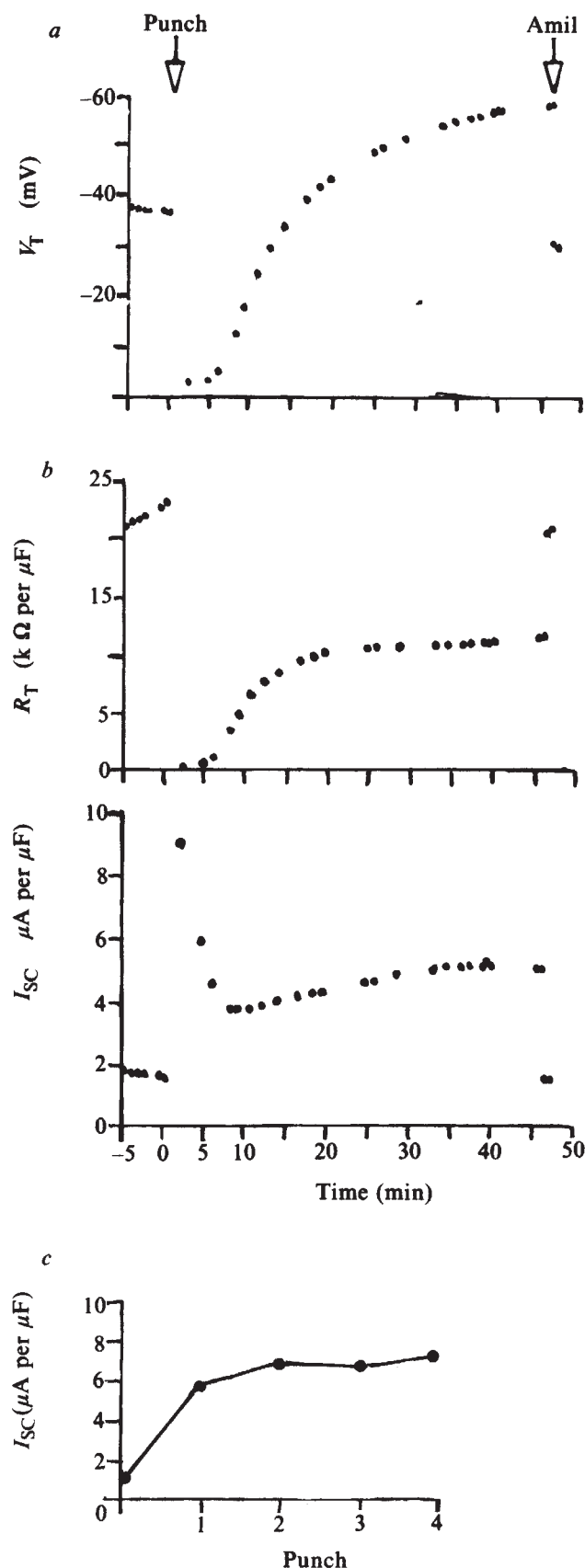


Fig. 2 a, Effect of 'punching' on V_T , R_T and I_{sc} . A single punch consisted of rapidly raising and lowering the mucosal solution 10 times (in the absence of a serosal solution). Total time, ~20 s. V_T and R_T were very reduced immediately after a punch. Over a period of 40 min both V_T and R_T increased, as did I_{sc} . V_T and I_{sc} exceeded pre-punch values and, as expected, R_T was reduced. The amiloride-sensitive current (Amil) was much larger than pre-punch values. b, Example of the number of punches required to bring the I_{sc} to a stable increased level. The mean value of five experiments was 2.2 ± 0.2 punches.

by a single inverse exponential $C_T(t) = C_T(0) e^{-kt}$ where $C_T(t)$ is the normalized capacitance at any time t after restoration of isosmotic solutions, and k is the proportionality constant. In four experiments, k had a value of 0.087 ± 0.015 ($\pm$ s.d.) min^{-1} , that is, capacitance had decreased to within 37% of control after 11 min.

The cytoplasmic vesicles, apical membrane and tight junctions and desmosomes are all interconnected by a dense microfilament network⁶. The exact role of this network is unknown but has been suggested to aid in mechanical stabilization of the apical membrane during distension, and to be involved in vesicle translocation^{1,2}. Disruption of this network should inhibit vesicle translocation during swelling promoted by hypo-osmotic solutions; we tested this by investigating the effects of colchicine⁷ (a microtubule blocking agent) and cytochalasin B⁸ (CB, a microfilament disrupting agent). Pre-incubation for 180 min with colchicine (0.1 mM) resulted in an increase ($17 \pm 4\%$) in capacitance, and a much more rapid onset of the capacitance change after exposure to hypo-osmotic solutions (Fig. 1b). The decrease after returning to the normal Ringer's followed the same time course as untreated bladders. Pre-incubation with cytochalasin B ($\sim 35 \mu\text{M}$ for 60 min) produced no change in the epithelial capacitance but it did inhibit the capacitance increase due to hypo-osmotic serosal solutions (Fig. 1c). This therefore indicates that increases in apical membrane area are dependent on intact microfilaments.

To determine whether the decrease in apical area after returning the epithelium to isosmotic Ringer's was also filament-dependent, the epithelium was first incubated for 60 min in hypo-osmotic Ringer's and then for a further 60 min in the presence of CB. The epithelium was then returned to the isosmotic Ringer's (in the absence of CB). As in the control studies, the capacitance decreased as a single exponential. However, the proportionality constant decreased to $0.046 \pm 0.002 \text{ min}^{-1}$ compared with $0.087 \pm 0.015 \text{ min}^{-1}$, thus the time to reach 37% of control was increased from 11 to 22 min. These data indicate that the microfilaments are necessary not only for exocytosis of vesicles but also for endocytosis. Microtubule-blocking agents seem to cause a partial disruption of the cell volume regulating system as capacitance changes were noted in the absence of hypo-osmotic solutions, and on exposure to a hypo-osmotic solution, capacitance increased more rapidly than for controls.

What is the role of the vesicles in electrolyte transport? Lewis and Willis⁴ reported that the Na^+/K^+ selective permeability ($P_{\text{Na}}/P_{\text{K}}$) of the amiloride pathways is 2.3:1 in control animals and increases to 9:1 in animals maintained on low Na^+ diets (that is, there is an increase in endogenous aldosterone level). In the following experiment we determined the selectivity of the newly inserted membrane in diet and control animals. We used a method involving mechanical perturbation of the epithelium, which maximized the insertion of vesicles into the apical membrane. Briefly, this method (termed 'punching') involved removing the solution from both mucosal and serosal chambers. Next, we rapidly filled and emptied the mucosal chamber of solution—this compresses the epithelium against a nylon mesh. Finally, we filled both chambers with normal bathing solution.

Figure 2 illustrates the effect of punching on transepithelial potential (V_T , referenced to the serosal solution), transepithelial resistance (R_T , normalized to capacitance) and short-circuit current (I_{sc} , calculated as V_T/R_T). V_T and I_{sc} were greater and R_T less than the respective values before punching. A significant finding was that punching caused no change in apical capacitance but significantly enhanced (from 0.4 ± 0.1 to $4.7 \pm 0.7 \mu\text{A per } \mu\text{F}$; $\pm$ s.e.m., $n = 10$) amiloride-sensitive Na^+ transport. The Na^+/K^+ selective permeability ($P_{\text{Na}}/P_{\text{K}}$), calculated using the method of Lewis and Willis⁴, was greatly increased (30:1) compared with the pre-punch value but was not significantly different between control and diet animals. The absolute apical membrane Na^+ permeability was, however, increased in diet compared with control animals. A possible explanation for the

difference in P_{Na}/P_K between pre-punched and punched preparations might be related to an as yet unknown property of urine (for example, proteolytic enzymes) to cause degradation of the selectivity properties of the amiloride pathways. How does punching cause an increase in Na^+ transport but not capacitance? We hypothesize that rapid filling and emptying of the mucosal chamber drives the apical membrane and cytoplasmic vesicles towards each other. On contact, the apical membrane may capture some vesicles as they 'kiss' onto the apical membrane. After the pressure pulse, the apical membrane rebounds, and because the vesicles are tethered by microfilaments, this results in a removal of some vesicles from the apical membrane. Evidence which partly supports such a mechanism is that pretreatment of the epithelium with CB followed by punching, does not elicit an increase in Na^+ transport.

Because these vesicles contain amiloride-sensitive Na^+ transport systems, we have devised a method for estimating the area of the apical membrane relative to that of the cytoplasmic pool. This method is based on the assumption that all the apical membrane and cytoplasmic vesicles are readily exchangeable. First, we 'punched' the epithelium to a constant (elevated) Na^+ transport rate, then we eliminated irreversibly all apical Na^+ pathways using the sulphhydryl-reactive agent *p*-chloromer-

curibenzene sulfonic acid (PCMBS). Then we punched the epithelium to a new (lower) Na^+ transport rate. By comparing the Na^+ transport rates before and after PCMBS treatment and punching, we were able to estimate the area ratios. The ratio of pool/apical area was 3.3 ± 1.2 , in good agreement with morphological studies².

This represents the first report of a physiological role for the cytoplasmic vesicles in normal functioning of the mammalian urinary bladder. These vesicles serve a dual role, first by allowing an increase in the storage volume of the urinary bladder without a concomitant alteration in the cellular volume. Thus, although the cells undergo dramatic geometric changes, there is a minimal alteration in intracellular ionic activities. Insertion and removal of the vesicles depends on an intact microfilament network.

Second and most fascinating, the vesicles contain Na^+ transport pathways whose density is increased by aldosterone. This phenomenon raises the possibility of membrane transport regulation by insertion and removal of vesicles containing the transport protein.

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Na^+ transport and flux ratio through apical Na^+ channels in toad bladder

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In many epithelia, the rate of $NaCl$ reabsorption is determined by the activity of a Na^+ transport system in the outer (apical) membrane. In frog skin and toad bladder, this system is thought to involve transmembrane pores through which Na^+ ions move down an electrochemical activity gradient^{1–5}. It has been shown, however, that increased intracellular Na^+ concentration leads to reduction in unidirectional Na^+ influx^{6,7}, raising the possibility that ions do not move through the channel independently. The Ussing flux ratio equation is one test for independence of passive ion movements⁸. In particular, single-filing of ions through long pores can account for effects such as reduction of unidirectional fluxes by ions on the opposite (*trans*) side of the membrane; this type of transport mechanism is characterized by flux ratio exponents >1 (refs 9–11). I have evaluated the flux ratio exponent (n') for the apical Na^+ channel in the toad bladder as the ratio of tracer permeability to electrical conductance at electrochemical equilibrium^{9–11}, that is, $n' = RT/F^2(G_{Na}/J_{Na})$, where J_{Na} and G_{Na} are, respectively, unidirectional flux and the conductance for Na^+ ions through the channel in the absence of net Na^+ movement. I report here values of n' of 1.15 ± 0.10 and 1.08 ± 0.07 for activities of 40 mM and 10 mM Na^+ , respectively, in the outer solution. This channel is therefore occupied by at most one Na^+ ion at these activities.

Urinary bladders from toads (*Bufo marinus*) were mounted in Lucite chambers and short-circuited as described previously¹². The serosal surface was bathed in KCl–sucrose medium to depolarize the basolateral membranes. In these conditions, the resistance and potential difference across the basolateral membranes fall sharply¹², and transepithelial impedance measurements have shown that almost all the transcellular resistance is located at the apical membrane¹³. Analysis of voltage transients showed that this condition is maintained

in the Na^+ -loaded state in the presence of ouabain (my unpublished results). Conductance was measured by displacing the transepithelial voltage by 10 mV and G_{Na} was calculated as the difference in transepithelial conductance obtained by replacing mucosal Na^+ with K^+ or, equivalently, by adding 10^{-4} M amiloride to the mucosal solution¹². In the Na^+ -loaded state, the amiloride-sensitive current–voltage (I – V) relationship becomes linear near $V = 0$ (ref. 12), so that G_{Na} is a reliable measure of conductance. All conductance measurements were corrected for solution resistance. The intracellular Na^+ ion activity $[Na^+]_i$ was estimated from the amiloride-sensitive short-circuit current (I_{Na}) and G_{Na} , using the constant-field equation. This is a rough estimate, and does not affect the other measured or calculated parameters.

J_{Na} was measured as the uptake of ^{22}Na into the cell from the mucosal solution during brief (15–20 s) exposures to the isotope, following the protocol of Biber and Curran¹⁴. Uptake was corrected for extracellular Na^+ using 3H -mannitol as a marker. I also made a small correction for amiloride-insensitive uptake (J_{Na} , corrected for extracellular Na^+ , in the presence of 10^{-4} M amiloride). In control conditions, uptake was found to be linear with time for at least 24 s, and J_{Na} was almost equal to I_{Na} , in agreement with previous reports^{7,14,15} (Fig. 1).

To achieve electrochemical equilibrium for Na^+ across the membrane, ouabain was added to the serosal medium to block Na^+ extrusion from the cell. As shown in Fig. 2, I_{Na} declined rapidly at first, and then more slowly. $[Na^+]_i$ rose from initial values of <5 mM to ~ 40 mM, and then remained fairly constant. G_{Na} increased initially, as expected for an increase in $[Na^+]_i$, and then fell, indicating a decline in Na^+ permeability.

When $[Na^+]_i$ reached 40 mM, the mucosal solution containing 80 mM $NaCl$ was replaced with one of 40 mM $NaCl$. Instant-

Table 1 Flux-ratio exponent (n') of apical membrane Na^+ channel

Mucosal Na^+ concentration (mM)	J_{Na} (pEq s ⁻¹)	G_{Na} (mS)	$n' [RT/F^2(G_{Na}/J_{Na})]$
10 ($n = 14$)	233 ± 49	0.92 ± 0.19	1.08 ± 0.07
40 ($n = 14$)	278 ± 67	1.09 ± 0.19	1.15 ± 0.10

Values represent mean $\pm$ s.e.m.

taneously, I_{Na} declined to near zero, and G_{Na} fell slightly, whereas $[Na^+]_i$ did not change. The residual I_{Na} , slightly negative in Fig. 2, was not significantly different from zero in either series of experiments. The absence of a Na^+ current in the presence of a Na^+ conductance demonstrated that electrochemical equilibrium had been achieved. At this time, G_{Na} and J_{Na} were measured, and n' was computed. In further experiments, a similar protocol was followed, except that shorter Na^+ loading periods were used to increase $[Na^+]_i$ to ~ 10 mM, and G_{Na} and J_{Na} were measured with 10 mM Na^+ in the mucosal solution. Mean computed values of n' were 1.15 at 40 mM Na^+ and 1.08 at 10 mM Na^+ (Table 1); neither value is significantly different from 1 ($P > 0.1$).

The results indicate that single-filing of ions does not occur to any appreciable extent at these Na^+ concentrations; the channel is occupied by at the most one Na^+ ion at a time. Single-filing therefore does not account for the decline in Na^+ permeability resulting from increased intracellular Na^+ observed here (Fig. 2) and by others^{7,8,16}. The slow fall in G_{Na} at constant $[Na^+]_i$ implies that the decline in permeability may be an indirect result of high intracellular Na^+ , perhaps mediated by changes in intracellular Ca^{2+} (refs 7, 17, 18).

Furthermore, the data indicate that the saturation of Na^+ influx with increasing mucosal Na^+ (refs 5, 14, 19) does not reflect carrier or shuttle-type kinetics, as these mechanisms, in general, show positive coupling between unidirectional fluxes, and flux-ratio exponents < 1 at concentrations at or above the dissociation constant for the carrier site. The results are consistent with those of Fuchs *et al.*⁵, Van Driessche and Lindemann², and Aceves and Cuthbert²⁰ who found that saturation

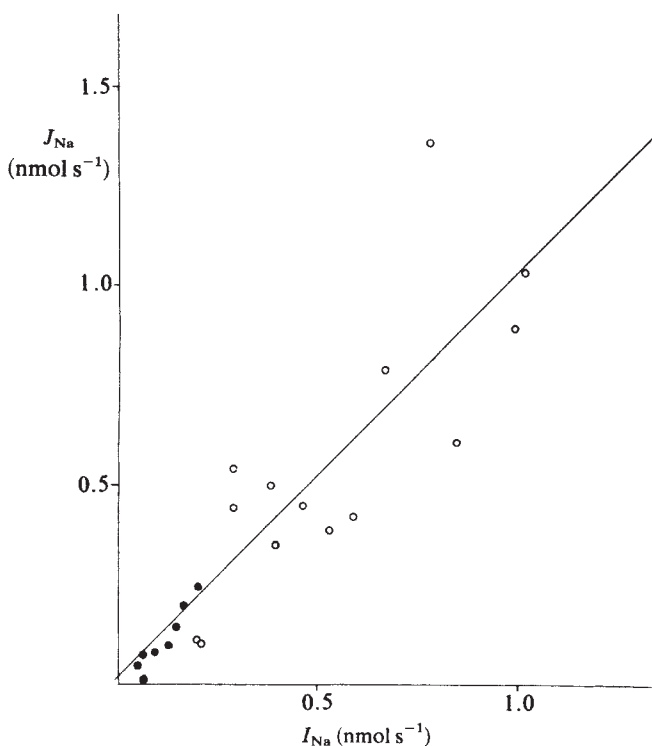


Fig. 1 Correlation of influx of Na^+ with net flux. Hemibladders were incubated with KCl-sucrose medium (composition (in mM): 85 KCl, 50 sucrose, 1 $CaCl_2$, 0.5 $MgSO_4$, 5 glucose, 3.5 K-phosphate, pH 7.7) on the serosal side and either 80 mM Na^+ concentration (composition (in mM): 108 NaCl, 7 KCl, 1 $CaCl_2$, 0.5 $MgSO_4$, 3.5 K-phosphate, pH 6.0) (O) or 10 mM Na^+ (replaced by choline) (●) on the mucosal side. Na^+ permeability was optimized at pH 6. Membrane area was 3 cm^2 . For measurements of J_{Na} , the mucosal solution was replaced with labelled solution of identical composition except for the addition of ^{22}Na ($5\text{ }\mu\text{Ci ml}^{-1}$, carrier-free), 3H -mannitol ($10\text{ }\mu\text{Ci ml}^{-1}$, 17 Ci mmol^{-1}) and 5 mM cold mannitol. The straight line represents a least-squares linear regression: $J_{Na} = 1.01 I_{Na} + 0.018$ ($r = 0.88$).

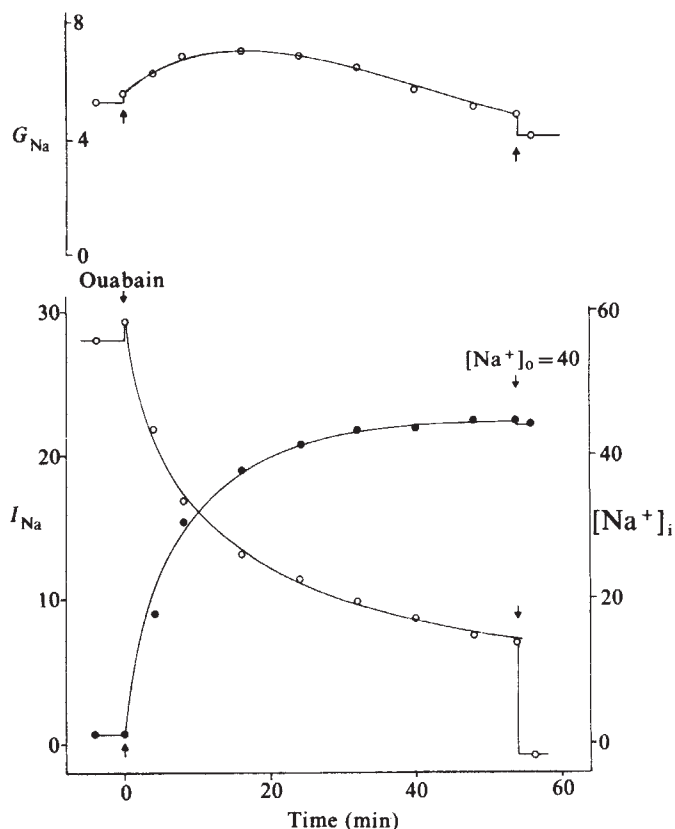


Fig. 2 Protocol for Na^+ -loading epithelial cells. Hemibladders were preincubated with KCl-sucrose medium on the serosal side, and 80 mM Na^+ on the mucosal side. Membrane area was 3 cm^2 . At time 0, ouabain (5 mM) was added to the serosal medium. G_{Na} (mS) and I_{Na} (μA) were calculated as the amiloride-sensitive conductance and short-circuit current, respectively. $[Na^+]_i$ (mM) was estimated from the constant-field equation assuming no membrane potential using: $G_{Na} = (F^2/RT)P_{Na}([Na^+]_o + [Na^+]_i)/2$ and $I_{Na} = (F)P_{Na}([Na^+]_o - [Na^+]_i)$ where P_{Na} is the Na^+ permeability and $[Na^+]_o$ and $[Na^+]_i$ are the Na^+ activities in the mucosal solution and in the cell, respectively. After 55 min, the Na^+ activity in the mucosal solution was rapidly decreased to 40 mM by replacement with K^+ . G_{Na} and J_{Na} were determined immediately afterwards, during the period in which $I_{Na} = 0$.

was accounted for by a decreased number of conducting channels, and not by saturation of single transporting sites.

Finally, deviations in transepithelial flux ratios from those predicted for passive, independent ion movement have been used to study active Na^+ transport in tight epithelia²¹. The results reported here imply that transport of Na^+ across the apical membrane Na^+ channel satisfies this condition for independence, and that the reported deviations of transepithelial flux ratio exponents from unity reflect the activity of the serosal Na^+ pump.

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Inhibitors of polyamine biosynthesis block human cytomegalovirus replication

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The antiviral activities of the few compounds available that are effective in the clinical treatment of virus infections are directed against nucleic acid synthesis^{1,2}. Attention has been drawn recently to the need to develop new strategies, based on specific inhibitors of other biopolymers with essential functions in virus replication². Polyamines may prove suitable targets, as these aliphatic bases are found in all animal cells, where they seem to have essential roles in the synthesis and stabilization of macromolecules^{3–5}. Here we describe how human diploid fibroblasts (MRC5 cells) infected with human cytomegalovirus (CMV) have elevated levels of polyamines, and the polyamine antimetabolite methylglyoxal bis(guanyldihydrazone) (MGBG) has potent antiviral activity. The growth of human CMV is also blocked by α -difluoromethylornithine (DFMO), a highly specific inhibitor of the initiation of polyamine biosynthesis. Cytochemical studies show this inhibitory effect is directed against early events in the virus replication cycle.

Infection of human diploid fibroblasts with human CMV is known to stimulate ornithine decarboxylase (EC 4.1.1.17) activity⁶. This enzyme catalyses the first step of polyamine biosynthesis, the formation of putrescine³. Another study has shown increased levels of [1,4-¹⁴C]putrescine uptake into MRC-5 cells infected with human CMV⁷. However, although radiolabelled polyamines (spermidine and spermine) were recovered at times from 24 h post-infection (p.i.), specific activities were not determined. We have now quantitatively analysed polyamine biosynthesis in MRC-5 cells infected at high multiplicity with human CMV strain AD169. The infected cells were recovered at 72 h p.i. and acid-soluble fractions were assayed for polyamines by the fluorometric measurement of their dansylated derivatives, after separation by TLC. The concentrations of spermidine and spermine in infected cells were 1.5- and 12.4-fold higher, respectively, than in uninfected control cells (Table 1). The higher specific activities of both polyamines in infected cells are consistent with the increased utilization of radiolabelled putrescine described in the earlier study⁷, showing clearly that infection with human CMV stimulates polyamine biosynthesis. The requirement for continued polyamine metabolism during human CMV replication is reflected in the antiviral effect of specific inhibitors of polyamine biosynthesis. Both MGBG, an inhibitor of spermidine and spermine biosynthesis, and α -methylornithine, a competitive inhibitor of ornithine decarboxylase, have been shown to prevent the production of infectious virus⁸. These results have encouraged further *in vitro* studies of the antiviral properties of MGBG in comparison with the nucleoside analogues 9-(2-hydroxyethoxymethyl) guanine (acyclovir) and 5-iodo-2'-deoxyuridine (IUdR), two drugs that have been used clinically for the treatment of human CMV infections^{1,9}. The inhibitory effect of each compound against eight different virus isolates was determined by plaque reduction methods (Table 2) and the median values (ED₅₀) for the antiviral effect of acyclovir (33.5 μ M), IUdR (5.3 μ M) and MGBG (0.6 μ M) demonstrate

Table 1 Spermidine and spermine content of uninfected and human CMV-infected MRC-5 cells

Cells	Polyamine	nmol per 2×10^{-7} cells	d.p.m. $\times 10^{-4}$ per 2×10^7 cells	Specific activity (mCi mmol ⁻¹)
Uninfected	Spermidine	75.8	4.11	0.25
	Spermine	18.5	0.82	0.19
Infected	Spermidine	110.0	16.15	0.66
	Spermine	230.0	42.34	0.83

MRC-5 cells were grown and maintained as described previously⁷. Confluent monolayers were mock-infected or infected with human CMV (strain AD169) using five plaque-forming units (PFU) per cell. All virus stocks were prepared from extracellular virus as described elsewhere¹⁶. At 24 h p.i. [1,4-¹⁴C]putrescine (116 mCi mmol⁻¹) was added to the maintenance medium to a final concentration of 0.1 μ Ci ml⁻¹ and the cells were recovered 48 h later. Polyamines were extracted and separated as their dansyl derivatives by TLC as described previously⁷. After extraction of the dansylated polyamines in dioxan, fluorescence relative to known amounts of appropriate standards were measured using a Locarte fluorimeter. Duplicate determinations were made and representative results are presented. The amount of radioactivity present in each extract was measured in a Packard liquid scintillation spectrometer.

the greater potency of the polyamine inhibitor. This effect on virus growth, which is readily reversed by exogenous spermidine (unpublished results), correlates with the reduction in polyamine biosynthesis in CMV-infected cells treated with MGBG⁸. However, this inhibitor is reported to have other effects on cellular functions which may be unrelated to polyamine metabolism¹¹.

DFMO is a highly specific inhibitor of polyamine metabolism with remarkably low toxicity^{12,13}. The compound is catalytically activated by ornithine decarboxylase, which it irreversibly inhibits¹⁴. The antiviral properties of DFMO were investigated by measuring its effect on the production of infective progeny virus. MRC-5 cells infected with human CMV were maintained in medium containing various concentrations of the compound, and infectivity titres were measured at 120 h p.i., a time after the exponential phase in the virus growth cycle⁷. There was a marked reduction in the amount of infective virus produced in cultures treated with 10 mM DFMO (see Fig. 1 legend). Lower concentrations had less effect on virus replication, and subsequent removal of the inhibitor from infected cultures restored virus growth. The replication of human CMV is characterized by the formation of intranuclear inclusions which are the sites of virus-specific DNA synthesis^{15,16}. These inclusions, visualized by acridine orange staining, develop progressively from 24 h p.i. to reach maximum size at 120 h p.i. (Fig. 1b,c). The production is inhibited completely by 10 mM DFMO (Fig. 1f), but with lower concentrations of the inhibitor the increase in virus yield is accompanied by development of the cytomegalic effect (Fig. 1d,e). These cytochemical studies show that the antiviral properties of DFMO are directed against early events in the CMV replication cycle associated with the synthesis or utilization of virus-specific DNA. Such results are consistent with the antiproliferative effects of DFMO described in other systems¹².

Table 2 A comparison of the antiviral effect (ED₅₀) of MGBG and two nucleoside analogues against eight strains of human CMV

Compound	Virus strains							
	AD169	Kerr	Rawles	BL	BR	LA	RE	WE
Acyclovir (μ M)	78	15	28	39	10	52	25	68
IUdR (μ M)	4.2	5.0	3.3	7.7	8.8	9.9	5.5	4.5
MGBG (μ M)	0.9	0.8	1.8	0.5	0.6	0.5	0.5	0.5

Three laboratory strains of CMV (AD169, Kerr, Rawles) and five clinical isolates with a low passage history were included in this study. In each experiment, dose response curves were constructed for acyclovir, IUdR and MGBG against the different strains of human CMV. A plaque reduction method was used to determine ED₅₀ values in a manner similar to that described by Collins and Bauer¹⁰. In brief, confluent MRC-5 cultures infected with about 100 PFU per culture were subsequently maintained in medium containing 0.5% agarose and supplemented with doubling concentrations of each compound. After 7–10 days incubation at 36°C, infected cultures were fixed in 5% formalin in phosphate-buffered saline and plaques were counted after staining with methylene blue. The ED₅₀ values were determined from plots of the percentage plaque reduction against the log₁₀ molar concentration of each compound.

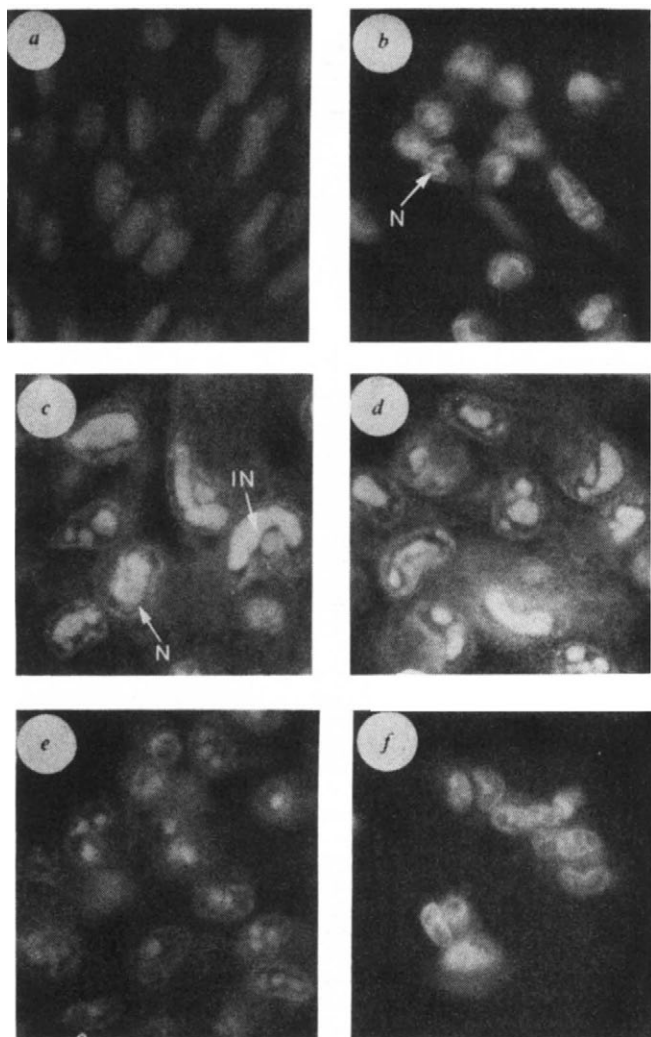


Fig. 1 The effect of α -difluoromethyl ornithine on the replication of human CMV strain AD169. MRC-5 cells (a) infected with human CMV (see Table 1) were maintained in medium supplemented with different concentrations of DFMO. Infected cells were recovered into fresh medium at the times indicated below for infectivity titration by plaque formation in MRC-5 cells. At the same times parallel, infected cultures were fixed *in situ* with absolute alcohol before staining with acridine orange¹⁵. Microscopic examination showed small rounded cells in all infected cultures at 24 h p.i. (b). Further virus growth resulted in the development of DNA-containing inclusions (IN) in the nuclei (N) of infected cells and, in addition, there was a marked increase in cell size (cytomegaly effect) by 120 h p.i. (c). In the presence of 10 mM DFMO, virus growth was inhibited and infected cells remained in the early, rounded form (f) but production of infective virus together with development of the intranuclear inclusions was seen with lower concentrations of the inhibitor (d, 2.5 mM; and e, 5 mM). Time p.i. (hours); b, 24; c, d, e and f, 120. Virus titre (PFU per 10^6 cells); b, $<10^2$; c, 2.2×10^6 ; d, 6.4×10^6 ; e, 7.5×10^5 ; f, 6.5×10^2 .

Further development of these observations by *in vivo* studies is limited by the lack of experimental animal models of the human disease. Polyamine inhibitors, however, have been used clinically as chemotherapeutic agents. MGBG has been used in the treatment of acute leukaemia¹⁷ with considerable benefit to the patient if the drug is monitored appropriately¹⁸. Clinical prospects are also good for the use of DFMO as an antiprotozoal agent¹⁹ and for the treatment of hyperproliferative states²⁰. In each instance the efficacy of chemotherapy may be related to inhibition of polyamine metabolism^{3,19,21}. Human CMV is the most important known infectious cause of mental retardation and a significant complication in immunocompromised hosts,

particularly allograft recipients²². No effective treatment of infections with this virus is currently available and there are certain limitations to prophylactic measures based on virus vaccines²³. Our observations show that polyamine metabolism is essential for the replication of human CMV and polyamine inhibitors may be suitable drugs for the treatment of CMV infections.

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Derivation of macrophage-like lines from the pre-B lymphoma ABL5 8.1 using 5-azacytidine

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Variation in the degree of methylation of DNA seems to be one mode of regulating gene expression in eukaryotic cells¹⁻⁷. The relationship between DNA demethylation and gene activation observed in globin⁴⁻⁶ and viral² genes, together with evidence that alterations in the degree of DNA methylation of a gene are heritable⁸, although not with 100% fidelity⁹, have suggested that this may be a mechanism of control of differentiation. Furthermore, exposure to the demethylating drug 5-azacytidine (5-AC) causes differentiation of 3T3 cells into striated muscle cells, chondrocytes and adipocytes⁷. Subsequent studies have shown that these effects are due to DNA demethylation¹⁰. In view of these observations, we have now attempted to modify several continuous B-cell lines with 5-AC. Following exposure of the pre-B lymphoma ABL5 8.1 to 5-AC, we have derived cloned cell lines which possess macrophage-like characteristics not expressed by ABL5 8.1. Similar macrophage-like cell lines were obtained in two independent experiments; they have been re-cloned and remain stable after 4 months of continuous culture.

ABL5 8.1 is a pre-B-cell tumour derived from a BALB/c mouse infected with Abelson virus¹¹. The line was originally

classified as B cell because of the presence of surface immunoglobulin¹². We can detect neither surface nor cytoplasmic immunoglobulin in these cells, but recently the classification of ABLS 8.1 was confirmed by demonstrating that rearrangement of an immunoglobulin variable region gene has occurred at both immunoglobulin heavy chain loci and at one κ chain gene locus (ref. 13 and S. Gerondakis, O. Bernard and E. Webb, in preparation), processes that seem unique to B-cell differentiation.

Cloned ABLS 8.1 cells were cultured at 2.5×10^5 cells per ml in the presence of various concentrations of 5-AC. Of cells cultured with $1 \mu\text{M}$ 5-AC and fed after 3 days with fresh medium again containing $1 \mu\text{M}$ 5-AC, only 20% of the initial number were present after 7 days. This population was cloned at limit dilution in liquid culture medium [Dulbecco's modified Eagle's medium (DME), 10% fetal calf serum, 5×10^{-5} M 2-mercaptoethanol (2-ME)]. Of 192 wells, 16 showed evidence of cell growth and these cells were expanded further and examined. Nine of the lines closely resembled ABLS 8.1 and on subsequent testing were indistinguishable from the parent cell line. The remaining seven cell lines were larger (mean cell volume 550 compared with $500 \mu\text{m}^3$ for ABLS 8.1) and were characteristically pleomorphic; ~5–20% of the cells had an elongate amoeboid or a stellate appearance and 1–2% adhered to the culture dishes. In contrast, ABLS 8.1 grew as a uniform population of round, non-adherent cells. Furthermore, unlike ABLS 8.1, the lines were able to grow in medium lacking 2-ME. All the lines were re-cloned in the absence of 2-ME in both agar gel culture and liquid culture; the morphological characteristics and ability to grow in the absence of 2-ME were heritable and occurred in all the clones.

One obvious possibility was that a contaminating cell type had been present in the initial cell population of ABLS 8.1 cells. This was excluded in three ways. First, the only tumours carried in tissue culture at the Hall Institute having characteristics resembling the lines putatively derived from ABLS 8.1 were the macrophage-like lines WEHI 3B and WEHI 265. Both, however, exhibited morphological differences from the 5-AC-treated cells and furthermore possessed characteristic metacentric chromosomes. ABLS 8.1 has no detectable chromosomal markers and is diploid. Chromosome spreads prepared¹⁴ from ABLS 8.1, WEHI 3B, WEHI 265 and the newly derived lines showed that ABLS 8.1, WEHI 3B and WEHI 265 had the expected karyotypes. None of the new cell lines had metacentric chromosomes and all had 40 chromosomes, which appeared identical to those of ABLS 8.1. The

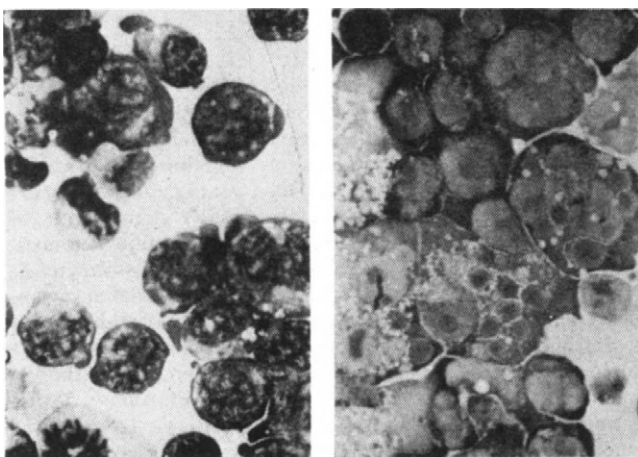


Fig. 1 5-AC-treated ABLS 8.1 cells are morphologically different from untreated ABLS 8.1 cells. Left, cytocentrifuge smear of ABLS 8.1 cells (May–Grünwald–Giemsa). Right, smear of a cloned line of cells derived from 5-AC-treated ABLS 8.1 cells. Three large vacuolated cells which have phagocytosed immunoglobulin-coated sheep red cells are shown.

Table 1 Comparison of the properties of ABLS 8.1 cells and lines derived using 5-AC

	ABLS 8.1	Cell line from first set of 5-AC-treated cultures	Cell line from second set of 5-AC-treated cultures
2-ME dependence	+	—	—
Phagocytosis:			
Latex*	1 ± 0.5	22 ± 3	15 ± 4
Ig-coated SRBCs†	0	12 ± 2	7 ± 3
Fc receptor‡	2 ± 1	63 ± 7	56 ± 8
Membrane Ig§	—	—	—
Membrane Thy-1§	—	—	—
Secreted Ig	—	—	—
MAC 1¶	0	6 ± 1.4	3 ± 1.1
Esterase #	±	++	++
Factor production:			
GM-CSF	—	—	—
TCGF	—	—	—
PSF	—	—	—
Presence of Ia antigen	—	—	—
Natural killer activity	—	—	—
Response to macrophage-activating factor**	—	—	—

GM-CSF, granulocyte-macrophage colony-stimulating activity, assayed by colony formation in agar culture²⁰. TCGF, T-cell growth factor activity, assayed as previously described²¹. PSF, P-cell factor which promotes growth of a mast cell progenitor; assayed as previously described²². Ia antigen was detected by incubating with a monoclonal antibody²³ at 4°C for 1 h, washing and adding fluoresceinated sheep anti-mouse immunoglobulin antibody for 30 min at 4°C. Natural killer activity was assayed on ⁵¹Cr-labelled YAC cells. Release of label into the supernatants was measured on a γ -counter and compared with background release and a positive control (nude mouse spleen cells).

* Cells were incubated overnight with latex particles and examined microscopically for evidence of phagocytosis.

† Sheep red blood cells (SRBCs) were washed in normal saline and resuspended to 5% (v/v) and mixed with an equal volume of 1:60 rabbit anti-SRBC antiserum. The cells were incubated for 1 h at 37°C, washed and resuspended to 2% v/v. Aliquots (50 μl) were added to 1 ml cultures of each cell line. Ig, immunoglobulin.

‡ Equal parts of 2% immunoglobulin-SRBCs (as in †) and cells at 5×10^6 per ml were incubated at 37°C for 15 min. The cells were centrifuged at 200g for 5 min and held at 4°C for a further 10 min. The suspension was stained with 0.1% crystal violet and rosettes counted.

§ The presence of membrane immunoglobulin and Thy-1 were tested for by staining with fluoresceinated rabbit anti-mouse immunoglobulin and fluoresceinated monoclonal anti-Thy-1.2 antibody (ref. 15) respectively.

|| Secreted immunoglobulin was assayed using a sensitive radioimmunoassay in 96-well microtitre trays coated with rabbit anti-mouse immunoglobulin antiserum.

¶ A monoclonal antibody specific for a macrophage cell surface antigen²⁴ obtained from Dr E. Handman, The Walter and Eliza Hall Institute.

Esterase stains were performed using the α -naphthyl butyrate method (Sigma). Scoring: ±, occasional cells (<10%) showing faint black granules; ++, many cells (>75%) showing unequivocally positive granules. In the conditions used thymocytes and peripheral blood lymphocytes were negative or faintly positive, but peritoneal exudate cells showed strong positive granules.

** Macrophage activation by concanavalin A-stimulated spleen cell supernatants was assayed as previously described¹⁶.

second way to exclude the possibility of contamination was to repeat the procedure with a new batch of ABLS 8.1 cells. Cloned ABLS 8.1 cells were cultured in agar with 5-AC as described above. To control for the possibility that 5-AC was not essential for the selection of a harder contaminant of variant line not requiring 2-ME, a parallel culture of 10^7 of the same clone of ABLS 8.1 cells was established in medium lacking both 5-AC and 2-ME. All these cells appeared dead after 3 days and no viable cells were detected over the ensuing 4 weeks of culture. Cells from the second batch of 5-AC-treated cultures

Table 2 Sodium butyrate induces increased differentiation of 5-AC-treated cells

	Control (%)	0.5 mM butyrate (%)	1 mM butyrate (%)
Morphological changes*	10 ± 3	96 ± 4	97 ± 5
Erythrophagocytosis†	4 ± 2	24 ± 6	7 ± 4
Esterase†	+	++	++

* The presence of granules and vacuoles similar to those seen in macrophages.

† Performed as described in Table 1 legend.

were removed at 0, 2 and 7 days and cultured at limit dilution in medium without 2-ME. No lines were obtained from the cells removed at day 0 or 2, but from day 7 cultures, 11 clones were obtained, 6 of which were morphologically similar to the 7 original lines. Both this and the original set of cell lines have been re-cloned and have remained stable for over 3 months of continuous culture. Final and compelling evidence that these cell lines were derived from ABL 8.1 was that the pattern of rearrangement of immunoglobulin J_H genes in one of the derived cell lines was identical to the characteristic pattern seen in ABL 8.1 (S. Cory, personal communication).

The granules and vacuoles present in the cell lines derived by 5-AC treatment of ABL 8.1 conferred on the cells the superficial appearance of macrophages (Fig. 1). We performed several tests which indicated that, unlike ABL 8.1, the derived cells possessed characteristics of macrophage cell lines (Table 1). The most impressive of these was phagocytosis of opsonized erythrocytes (Table 1, Fig 1) which was not seen in control ABL 8.1 cells and seems to be a unique property of macrophages. Other strong supporting evidence was the presence of esterase-positive granules and the presence on a significant percentage of cells of an antigen detected by a macrophage-specific monoclonal antibody (Mac-1)¹⁵. As may be expected from the heterogeneity inherent even in cloned cell lines, these properties were not expressed by all cells in the population. Other properties such as the expression of Ia antigens and Fc receptors are less useful discriminants, as they are shared by both B lymphocytes and macrophages. Interleukin-1 (IL-1) production and the ability to present antigen are still being investigated. The cells did not produce detectable colony-stimulating factors (or T-cell growth factor or P-cell stimulating factor). They were not cytotoxic to a good target for natural killer cells (YAC cell line); nor did the derived cells lyse another tumour target (P815) in the presence of medium conditioned by concanavalin A-stimulated spleen cells known to contain macrophage activating factor¹⁶.

In attempts to increase the proportion of more differentiated cells, agents known to induce differentiation were added to the cultures. Of those tested, dimethyl sulphoxide (0.5–10% v/v), actinomycin D (0.1–10 $\mu\text{g ml}^{-1}$), and tetradecanoyl phorbol acetate (1–100 ng ml^{-1}) or combinations of these had no effect at concentrations which did not severely affect viability. On the other hand, sodium butyrate (0.1–1 mM), while reducing the rate of cell division, did not obviously reduce viability in 3–5-day cultures. At concentrations of 0.3–1 mM, increasing proportions of differentiated cells were observed over 2–3 days (Table 2). After 3 days, in the presence of 0.5–1 mM sodium butyrate morphological changes such as the presence of granules and vacuoles had occurred in almost all the cells. In parallel experiments using ABL 8.1, the presence of sodium butyrate was associated with the appearance of a small percentage (14%) of larger cells having an increased number of vacuoles. However, these cells were clearly distinguishable from those seen in 5-AC-treated cells cultured with butyrate, as they lacked granules and did not phagocytose erythrocytes.

We conclude that the demethylating drug 5-AC has induced heritable morphological and functional changes in the pre-B-cell line ABL 8.1 characterized by the expression of

macrophage-like features. The percentage of cells showing these properties was markedly increased by sodium butyrate which slowed the rate of cell division. This effect seemed to require prolonged exposure to the drug, which may suggest that a definite sequence or combination of demethylation steps is required to induce the observed changes. The finding that the only variants were macrophage-like cells may be explained in two ways. First, the culture conditions used might select for the survival of such cells. A second more interesting possibility is that this result reflects the origin of both pre-B lymphocytes and macrophages from a common progenitor and that the demethylation activates certain genes which cause ABL 8.1 to differentiate along a programmed or favoured path into a closely related, but functionally different cell. A close relationship between the lymphocyte and macrophage lineages is supported by evidence that the immunoglobulin μ -chain constant region gene is transcribed in myeloid lines and macrophages¹⁷. Although rearrangement of immunoglobulin variable (V) region genes has not been found in macrophage-like tumour lines examined so far, transcription of the μ gene in these cell lines may imply that the region of the genome encoding immunoglobulins undergoes some activation in a common precursor of B cells and macrophages. The fact that rearrangement of V genes has occurred on both chromosomes in ABL 8.1 suggests that in this respect, ABL 8.1 is at a B cell-specific differentiation stage beyond this putative common precursor. Nevertheless, given some activation of the immunoglobulin-coding region of the genome in macrophages^{17,18} and, presumably, their precursors, it is possible that the reciprocal situation—some activation of macrophage-specific genes—also occurs in pre-B cells and accounts for the tendency for perturbation of the ABL 8.1 genome by 5-AC to result in cells having macrophage characteristics. We are also investigating whether different cloning conditions could allow us to grow out other related haematopoietic cells.

Although experiments of this nature may provide useful information about the genetic mechanisms of gene expression, at the present stage of knowledge, extrapolation from the observed changes in phenotype to conclusions about physiological sequences of differentiation must be cautious. However, given the minimal amount known concerning the number and sequence of differentiation steps between multipotential haematopoietic stem cells and B cells¹⁹, new approaches such as this may well yield useful clues to normal differentiation events.

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HLA-DR light chain has a polymorphic N-terminal region and a conserved immunoglobulin-like C-terminal region

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The major histocompatibility complex (MHC) is a genetic region originally defined by graft rejection and now known to encode at least three classes of molecules which have important roles in the immune system. The MHC class I and class II antigens are both polymorphic two-chain cell-surface glycoproteins which are recognized by T lymphocytes. However, they are generally recognized by different subsets of T cells and have different functions, different tissue distributions and, by all available evidence, different structures¹⁻⁴. Much is known about the detailed structure of the class I antigens (HLA-A, B, C in human; H-2K,D,L in mouse)⁵⁻⁷. The 44,000 (44K)-molecular weight (M_r) heavy chain consists of an amino-terminal extracellular region composed of three 10K M_r (90 amino acid) domains, a small hydrophobic membraneous segment and a small hydrophilic intracellular carboxy-terminal domain. The two amino-terminal domains are polymorphic, bear the carbohydrate and have no sequence homology with immunoglobulin. The third domain, closest to the membrane, and the 11.6K light chain (β_2 -microglobulin) are highly conserved and have strong sequence homology with immunoglobulin. The structure of class II antigens (DR, DC1 in human; I-A, I-E in mouse) is much less well understood. Previous experiments involving papain proteolysis of native DR antigen have shown that both the light and heavy chains have a large glycosylated amino-terminal extracellular region, a small hydrophobic membraneous region and a small carboxy-terminal hydrophilic region⁸. We have now applied limited proteolysis to demonstrate that the extracellular region of the light chain consists of two domains, each with a disulphide loop. The amino-terminal domain bears the carbohydrate and is polymorphic, while the carboxy-terminal domain is relatively conserved and has significant amino acid sequence homology with immunoglobulin. These results suggest a new picture of class II antigens indicating strong structural similarities to both class I antigens and immunoglobulins.

The B lymphoblastoid cell line WT51 (derived from an individual homozygous for HLA-DR4) was metabolically labelled with ³⁵S-cysteine. A detergent-solubilized cell lysate was prepared and treated with various proteases, the DR antigens immunoprecipitated with the rabbit xenoserum anti-p23, 30 and the precipitates analysed by SDS-polyacrylamide gel electrophoresis and fluorography. Before proteolysis, the DR4 antigen consists primarily of a 34K M_r heavy chain and a 29K M_r light chain (Fig 1a, lane 1). Proteolysis with chymotrypsin (Fig. 1a, lane 2) and with certain other proteases generates three major bands: 33K, 15.7K and a doublet of 12.6K and 13.2K. Proteolysis with trypsin or endoproteinase Lys-C (Fig. 1a, lanes 3, 4) generates a different pattern also with three major bands, of M_r 33K, 17K and 11.3K. Proteolysis with clostripain (Fig. 1a, lane 5) generates only two new bands at 33K and 27K.

Serial proteolysis with papain showed that the 33K and 27K bands represent the DR heavy and light chains with the carboxy-terminal hydrophilic regions removed. These fragments were

generated by every protease tested, albeit with different efficiencies and at slightly different sites⁹. Only papain in very large amounts removes the hydrophobic regions⁸, but the light chain could be further cleaved into large fragments by two classes of protease as shown in Fig. 1. Several experiments with DR4 antigen (including amino acid sequencing, see below) demonstrated that the larger bands (N_{ct} , 15.7K; N_t , 17K) are amino-terminal fragments of the light chain and the smaller bands (C_{ct} , 12.6K and 13.2K; C_t , 11.3K) are carboxy-terminal fragments of the light chain. Serial proteolysis with papain showed that the 13.2K band represents the entire carboxy-terminal chymotryptic fragment, while the 12.6K band represents the carboxy-terminal chymotryptic fragment lacking the hydrophilic carboxy-terminal peptide. Similar results were obtained with DRw6 antigen⁹.

Endoglycosidase digestion has demonstrated that the DR heavy chain bears two N-linked glycans of M_r ~3K, whereas the light chain bears only one¹⁰. The antibiotic tunicamycin inhibits the glycosylation of DR antigens during biosynthesis (Fig. 1b, lanes 1, 3)¹¹. Chymotryptic digestion of lysates from tunicamycin-treated cells generates two bands (26K and 13K) which immunoprecipitate with anti-p23,30, instead of the expected three bands (Fig. 1b, lanes 2, 4). Other experiments (not shown) using endoglycosidase D and antisera specific for either DR heavy or light chain demonstrate that the 26K fragment is the chymotryptic product of the non-glycosylated heavy chain, while the 13K band contains both the carboxy-terminal and the non-glycosylated amino-terminal fragments of the light chain. Thus, the amino-terminal chymotryptic fragment of the light chain bears the carbohydrate; also, the chymotryptic site is roughly in the middle of the chain. These results were confirmed with Drw6 antigen⁹.

The mobility of denatured light chain in SDS-polyacrylamide gel electrophoresis depends markedly on whether or not it is reduced¹². This mobility shift is greater than that of β_2 -microglobulin (one disulphide loop), the heavy chain of HLA-A and -B antigens (two disulphide loops) and the heavy chain of DR antigens (one disulphide loop) (Fig. 2, lanes 1-4). Amino acid analyses had suggested that both the light and heavy chains of DR contained three cysteines^{8,12}. However, the heavy chain could be labelled by mild reduction and alkylation, while the light chain could not⁸. Moreover, careful counting of cysteines in material from the lymphoblastoid line JY (DR4,w6) demonstrated that the heavy chain has one disulphide loop and one free sulphhydryl, whereas the major light chain has four cysteines in two disulphide loops with no free sulphhydryls⁹. It was thus of interest to determine the location of these disulphide loops in the light chain.

Lysates from ³⁵S-cysteine-labelled cells were proteolysed with either chymotrypsin or trypsin, immunoprecipitated with anti-p23,30, boiled in SDS sample buffer with or without 2 mM dithiothreitol (DTT) and analysed by SDS-gel electrophoresis. Both the amino-terminal and carboxy-terminal chymotryptic fragments contain comparable amounts of ³⁵S-cysteine and have a mobility shift equivalent to that of β_2 -microglobulin (Fig. 2, lanes 5, 6). Thus, each chymotryptic fragment of the light chain contains a disulphide loop. This fact was confirmed by the trypsin cleavage. Electrophoresis in reducing conditions yielded the expected 17K amino-terminal and 11.3K carboxy-terminal tryptic fragments (Fig. 2, lane 7). Electrophoresis in non-reducing conditions yielded a band at approximately the mobility of the unreduced light chain (24K; Fig. 2, lane 8), corresponding to the amino-terminal and carboxy-terminal tryptic fragments still linked together by a disulphide bond. Thus, the trypsin cleaves between the two cysteines of the second disulphide loop, consistent with the fact that the amino-terminal fragment contains much more ³⁵S-cysteine than the carboxy-terminal tryptic fragment (Fig. 2, lane 7).

To define the cleavage fragments better, automated amino-terminal sequencing was carried out on chymotrypsin fragments from WT51 cells biosynthetically labelled with various amino acids (Fig. 3) The partial amino-terminal sequence for the 15.7K

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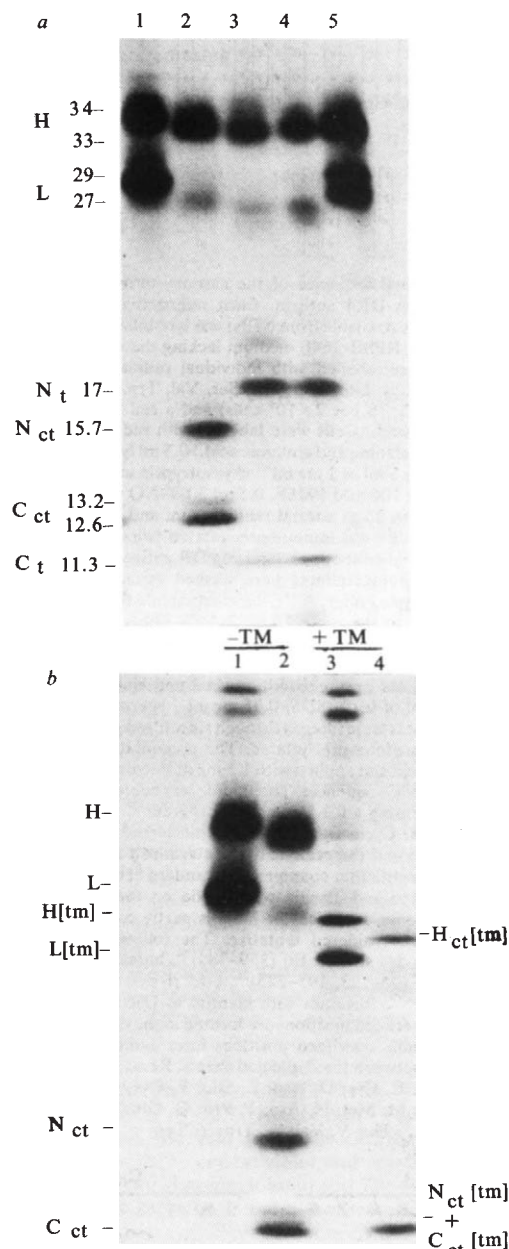


Fig. 1 SDS gel of DR antigen cleaved by various proteases. Except where noted, cells were labelled as follows. 2×10^6 WT51 cells were preincubated for 30 min at 37°C in 2 ml cysteine-free RPMI-1640 medium supplemented with 10% dialysed fetal bovine serum (Gibco), 100 U ml^{-1} penicillin and 0.1 mg ml^{-1} streptomycin. Cells were centrifuged and resuspended in the same medium supplemented with $1 \text{ mCi } ^{35}\text{S-cysteine}$ (NEN) and then incubated for 20 h at 37°C . For tunicamycin-treated cells, $4 \mu\text{g ml}^{-1}$ tunicamycin (TM) was included in both the preincubation and incubation media. Labelled cells were collected by centrifugation and incubated for 30 min on ice in 1 ml lysis buffer containing 2% Nonidet-P40 (NP40), 20 mM Tris-HCl pH 8, 1 mM MgCl_2 , 150 mM NaCl and 0.1 mM phenylmethylsulphonyl fluoride (PMSF). Subcellular debris was removed by centrifugation in an Eppendorf centrifuge for 10 min at 4°C and then the lysate was frozen at -80°C until use. Except where noted, proteolysis was carried out as follows. One volume of lysate (usually 5 μl) was incubated with 10 volumes (50 μl) of buffer or protease at 37°C for 1 h and then proteolysis was stopped by incubation on ice for 30 min with added proteolysis inhibitor. The serine proteases chymotrypsin (2 mg ml^{-1} , 40 U per mg, Worthington), diphenylcarbamylchloride-treated trypsin (2 mg ml^{-1} , 8,000 U per mg, Sigma), endoproteinase Lys-C ($0.2 \text{ mg protease ml}^{-1}$, 0.3 U per mg, Boehringer-Mannheim) in 40 mM Tris-Cl pH 8, 100 mM NaCl, 10 mM CaCl_2 were halted by 0.2 volumes (1 μl) 100 mM PMSF in absolute ethanol and the sulphhydryl proteases papain (0.01 mg ml^{-1} , 25 U per mg, Millipore) and clostripain (2 mg ml^{-1} , 40 U per mg, Boehringer-Mannheim) in 10 mM Tris-Cl pH 8, 0.1 mM EDTA, 1 mM DTT were halted by 5 volumes (25 μl) 25 mM iodoacetamide in water. Forty volumes (200 μl) of NET-NO (50 mM Tris-Cl pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mg ml^{-1} ovalbumin) were added and the solution precleared with normal rabbit serum and formalin-fixed *Staphylococcus aureus* Cowan I strain (Staph A), immunoprecipitated with the rabbit xeno serum anti-p23,30¹⁸ and Staph A, and washed as described previously¹⁹. Immunoprecipitates were eluted by boiling in sample buffer (200 mM Tris-Cl pH 8.8, 10% glycerol, 0.1% bromophenol blue, 5 mM EDTA, 2% SDS, 5% 2-mercaptoethanol) and analysed by electrophoresis through 7–15% polyacrylamide gradient gels (using Laemmli buffers²⁰ with 2 mM EDTA added) and fluorography²¹. In a, 5 μl lysate was incubated for 1 h at 37°C with 50 μl buffer or protease and then proteolysis was halted on ice with either 1 μl 100 mM PMSF or 25 μl 25 mM iodoacetamide. 200 μl NET-NO was added and the sample was precleared, immunoprecipitated with anti-p23,30 and analysed. Lane 1, no protease; lane 2, chymotrypsin; lane 3, trypsin; lane 4, endoproteinase Lys-C; lane 5, clostripain. Numbers on the left represent M_r ($\times 10^{-3}$). b: Lane 1, DR, no protease; lane 2, DR, chymotrypsin; lane 3, tunicamycin DR, no protease; lane 4, tunicamycin DR, chymotrypsin. H, DR heavy chain; L, DR light chain; N_t , amino-terminal tryptic fragment; N_{ct} , amino-terminal chymotryptic fragment; C_{ct} , carboxy-terminal chymotryptic fragment; C_t , carboxy-terminal tryptic fragment; tm, tunicamycin.

amino-terminal fragment is homologous to published amino-terminal sequences of human DR and murine 1-E light chains. The partial amino-terminal sequence of the 13K carboxy-terminal fragment shows no homology with the amino-terminus of the whole molecule. However, quite striking homologies are evident with the first cysteine region of the second disulphide loop ($\alpha 3$) of class I antigens (such as HLA-A and B), β_2 -microglobulin and immunoglobulin constant regions. On inspection, the best homology occurs with the C_H3 domains of IgG (in descending order C_H3 , C_H1 , C_L , C_H2 and V).

The amino acids which are conserved or invariant within immunoglobulin domains are known to correspond to particular structural features^{13,14}. The portion under consideration here includes the second and third antiparallel β -strands (found in the four-strand (fx) and three-strand (fy) sheets, respectively) and the loop between them (b2). In this region, the highly conserved residues (numbered from the site homologous to the chymotrypsin site in DR light chain) include the invariant cysteine-8 (involved in the disulphide loop), invariant tryptophan-22 (which shields the disulphide bond), highly conserved proline-15 (found in the bend) and the conserved alternating hydrophobic amino acids in the antiparallel β -strands

(whose side chains fill the space between the two β -pleated sheets of the immunoglobulin domain: leucine-6, cysteine-8, valine-10, phenylalanine-13, isoleucine-18, valine-20, tryptophan-22). Every conserved or invariant residue in this region of the C_H3 domain of IgG myeloma Eu is identical to that of the carboxy-terminal chymotryptic fragment of the DR light chain. These same residues are conserved or identical in the second disulphide loop region of HLA-A and -B and in β_2 -microglobulin, except that β_2 -microglobulin has leucine-22 instead of the invariant tryptophan. Several other residues determined are conserved. In the aligned sequence, the chymotryptic site is located in the middle of the loop between the first and second antiparallel β -strands (b1). The trypsin site is likely to be located at lysine-30 (the first endoproteinase Lys-C-sensitive site after the chymotrypsin site), which is in the loop between the third and fourth anti-parallel β -strands (b3). These sites are at the same end of the immunoglobulin domain^{13,14}, and in the DR light chain, that end must be exposed to solvent. In fact, the chymotrypsin site is accessible even in membrane-bound DR antigens (J.K., unpublished).

In class I antigens of both human and mouse, the regions with homology to immunoglobulin (β_2 -microglobulin and the second disulphide loop of class I antigens) are invariant or highly conserved⁵⁻⁷. It was of interest to determine whether this is also true for the DR light chain, which is the polymorphic chain^{15,16}. DR antigens from a number of cell lines (homozygous for DR1-8) were proteolysed with chymotrypsin, immunoprecipitated with anti-p23,30 and analysed by isoelectric focusing (IEF). By this criterion, the amino-terminal chymotryptic fragments of the light chain vary considerably (Fig. 4, lanes 1-8), whereas the carboxy-terminal fragments are quite conserved (Fig. 4, lanes 9-16). Whether the slight variability

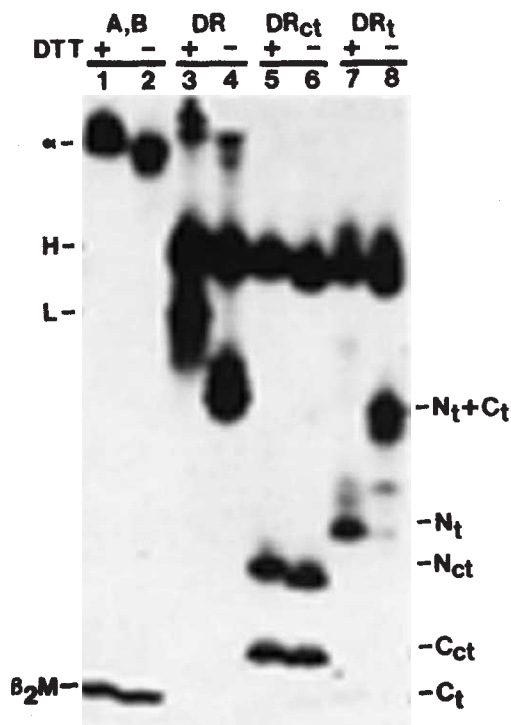


Fig. 2 SDS gel of reduced and nonreduced proteolytic fragments of DR antigen. As described for Fig. 1, samples were proteolysed and immunoprecipitated with anti-p23,30 or w6/32 (a monoclonal antibody which recognizes HLA-A, -B and -C antigens)²². The immunoprecipitates were boiled in sample buffer with either no reducing agent or 2 mM DTT and analysed by SDS-gel electrophoresis and fluorography. Lane 1, no protease, w6/32, DTT; lane 2, no protease, w6/32, no DTT; lane 3, no protease, anti-p23,30, DTT; lane 4, no protease, anti-p23,30, no DTT; lane 5, chymotrypsin, anti-p23,30, DTT; lane 6, chymotrypsin, anti-p23,30, no DTT; lane 7, trypsin, anti-p23,30, DTT; lane 8, trypsin, anti-p23,30, no DTT. α , HLA-A,B heavy chain; β_2M , β_2 -microglobulin.

in carboxy-terminal fragments is due to polymorphism at the site of proteolysis or to limited polymorphism elsewhere (as in mouse β_2 -microglobulin¹⁷ or human α 3⁶) is unclear. However, at least in the case of DRw6 from cell line LB and DR4 from cell line WT51, the chymotrypsin sites are homologous⁹.

Protease cleavage of the native DR antigen has defined four domains for the DR light chain: a polymorphic glycosylated amino-terminal domain with a disulphide loop but no sequence homology with immunoglobulin, a relatively conserved domain with a disulphide loop and strong sequence homology with immunoglobulin, a hydrophobic region and a carboxy-terminal hydrophilic region. This organization of domains, as defined by protease cleavage, has been found in light chains from every class II antigen tested—human DR antigens (specificities 1–8), human DC1 antigens, mouse I-A and I-E antigens⁹. In fact, this general domain organization also extends to class II heavy chains. Papain proteolysis studies⁸ and amino acid sequence data derived from genomic clones (A. Korman, personal communication) demonstrate that the DR heavy chain also has a four-domain structure: an amino-terminal domain having no structural homology with immunoglobulin, a second domain having striking sequence homology with immunoglobulin, a hydrophobic region and a carboxy-terminal hydrophilic region. The model based on these data (Fig. 5) allows homologous pairing between the domains of the two chains.

Like the class I antigens, the extracellular region of class II antigens consists of four domains: two having sequence homology to immunoglobulin and two without. It would be surprising if the gross three-dimensional structure and the function at the molecular level were different for the class I and II antigens. Another intriguing point is that the organization of domains in both the DR light and heavy chains is strikingly like that of a class I antigen heavy chain lacking one of the two

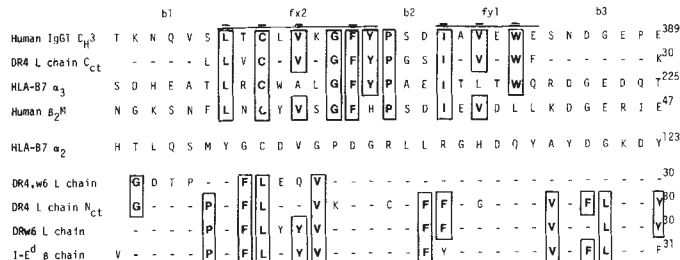


Fig. 3 Amino-terminal sequence of the carboxy-terminal chymotryptic fragment of the HLA-DR4 antigen. Each radioactive amino acid (the highest specific activity available from NEN) was lyophilized to near-dryness and then dissolved in RPMI-1640 medium lacking the appropriate amino acid. WT51 cells were labelled with individual radioactive amino acids—Ala, Cys, Phe, Ile, Lys, Leu, Met, Pro, Ser, Val, Trp, Tyr, 1–3 mCi ³H, 100 μ Ci ¹⁴C, 1–2 mCi ³⁵S per 2×10^6 cells and a cell lysate prepared as outlined in Fig. 1 legend. (Cells were labelled with radioactive alanine in medium lacking both alanine and glutamic acid.) 0.5 ml lysate was incubated for 1.5 h at 37 °C with 5 ml of 2 mg ml⁻¹ chymotrypsin and then proteolysis was halted with 50 μ l 100 mM PMSE. 0.5 ml NET-NO was added and the lysate was cleared with 25 μ l normal rabbit serum and 300 μ l 10% Staph A. The precleared lysate was immunoprecipitated twice with 20 μ l ascites of LB3.1 (a monoclonal antibody recognizing DR antigen) and 250 μ l 10% Staph A. The immunoprecipitates were washed as usual and eluted by boiling into 90- μ l sample buffer. A ¹⁴C-labelled sample and or a ³H-labelled sample were applied to the same 20 cm 7–15% polyacrylamide gradient SDS gel as a ³⁵S-cysteine-labelled sample. The presence of the ³⁵S-cysteine allowed the bands to be located by wet gel autoradiography after electrophoresis¹⁶. Gel slices were excised, crushed and eluted by shaking for 24 h at 4 °C into 1 ml of 0.1% SDS, 0.35 mg ml⁻¹ sperm whale myoglobin (Beckman). The eluates were filtered through siliconized glass wool, lyophilized to 200 μ l and acetone-precipitated. The precipitate was dissolved in 300 μ l 50% formic acid and applied with 1.5 mg of Polybrene to the spinning cup of a Beckman 890C sequencer. The sample was subjected to automated Edman degradation using a 0.1 M Quadrol program²³, with double-coupling at the first step. Cycle fractions were transferred to counting vials, dried under nitrogen and the radioactivity determined using Aqualos and a Beckman liquid scintillation counter with standard ³H and ¹⁴C windows. Assignments of serine and glycine were made on the basis of samples labelled with ¹⁴C-serine, in which the label is partly converted to glycine and should thus be considered tentative. The following residues from published sequences are shown: Eu (359–389)²⁴, human β -microglobulin (17–47)²⁵, HLA-B7 (93–123, 195–225)²⁶, I-E β -chain (1–31)²⁷ and DR light chains (1–30)^{12,27}. Residues with identity to DR chymotryptic fragments are boxed. Overlined positions are located in the designated β -strand of Eu C α 3 and double overlined positions have amino acid side chains which fill the space between the β -pleated sheets. Residues are in the single letter code: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr.

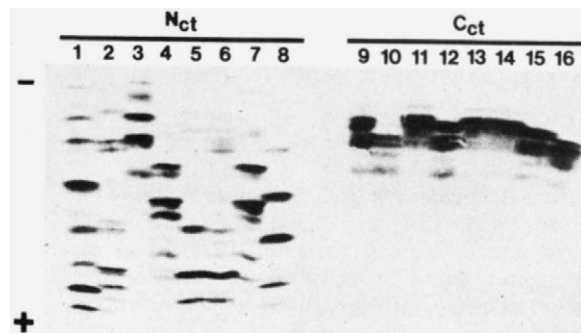


Fig. 4 IEF gel of chymotryptic fragments of DR antigens from various cell lines. Cells were labelled with ³⁵S-cysteine, proteolysed with chymotrypsin and immunoprecipitated with anti-p23,30 as described in Fig. 1 legend. After SDS-gel electrophoresis of samples, bands across all the lanes at the mobilities of the amino-terminal and carboxy-terminal fragments were excised (using dansylated β -lactoglobulin and myoglobin as markers). These gel slices were prepared for IEF by soaking in 8 M urea, 2% NP40, 0.2% pH 9–11 ampholines for 1 h at room temperature. The gel slices were applied to the acid end of a polymerized IEF gel containing 8 M urea, 2% NP40, 7% acrylamide, 0.19% N,N'-methylenebisacrylamide, 2% (w/v) ampholines (LKB) (pH 3.5–10, 4–6, 5–7, 6–8, 7–9, 9–11; 2:1:1:1:1) and focused at 1,000 V for 8 h at 7 °C using an LKB Multiphor 2117 flatbed gel apparatus¹⁵. Lanes 1–8, ³⁵S-cysteine-labelled DR, amino-terminal chymotryptic fragments; lanes 9–16, ³⁵S-cysteine-labelled DR, carboxy-terminal chymotryptic fragments. Lanes 1 and 9, MAJA (DR1,1); lanes 2 and 10, PGF (DR2,2); lanes 3 and 11, LKT (DR3,3); lanes 4 and 12, WT51 (DR4,4); lanes 5 and 13, MICH (DR5,5); lanes 6 and 14, LB (DRw6,w6); lanes 7 and 15, MANN (DR7,7); lanes 8 and 16, MADURA (DR8,8).

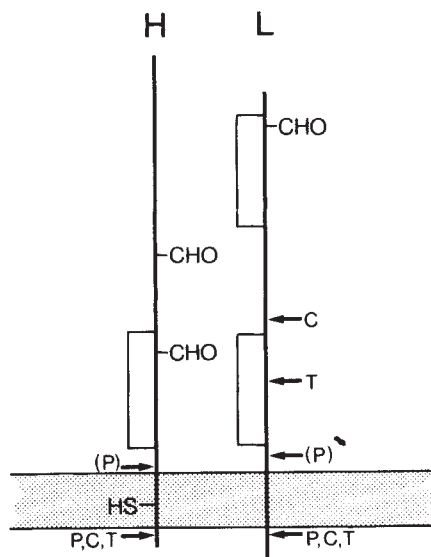


Fig. 5 Proposed model of HLA-DR antigen. H, heavy chain; L, light chain; P, papain site; C, chymotrypsin site; T, trypsin site. Thin lines represent disulphide bonds; -SH, free sulphhydryl. Stippled area represents the membrane.

amino-terminal domains which do not have sequence homology with immunoglobulin. It therefore seems likely that both class I and II antigens descended from a common ancestral gene containing an immunoglobulin-like region.

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Note added in proof: Since the submission of this paper, a preliminary report of the partial sequence of a human class II antigen has appeared²⁸. The sequence is consistent with the data presented here.

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Expression of lymphocyte surface IgE does not require switch recombination

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Immunoglobulin heavy (H) chains are composed of a variable (V_H) and a constant (C_H) region. The latter is encoded respectively by eight distinct genes for the classes and subclasses in mice— C_{μ} , C_{δ} , $C_{\gamma 3}$, $C_{\gamma 1}$, $C_{\gamma 2b}$, $C_{\gamma 2a}$, C_{ϵ} and C_{α} genes—arrayed in that order on the chromosome¹. During differentiation of a single B lymphocyte, a given V_H region is first expressed as a μ chain, followed by the switch of the C_H region to other classes such as δ , γ , ϵ and α . The molecular genetic basis for this phenomenon, called heavy chain class switch, has been elucidated recently by cloning and characterization of immunoglobulin genes of mouse myelomas secreting various classes of immunoglobulin^{2–4}. By this model, DNA rearrangement, called S–S recombination, brings a V_H gene, located originally 5' to the C_{μ} gene, close to another C_H gene by deletion of an intervening DNA segment^{5–9}. The S–S recombination occurs between S regions located in the 5' flanking region of each C_H gene. The nucleotide sequences of S regions comprise tandem repetitive sequences sharing short common sequences^{10–14}. In contrast, the expression of surface IgD in $\mu^+\delta^+$ lymphomas and in normal $\mu^+\delta^+$ lymphocytes seems not to involve DNA rearrangement in the region between the C_{μ} and C_{δ} genes^{15,16}. The simultaneous expression of the C_{μ} and C_{δ} genes with a single V_H gene may be mediated by two alternative routes of RNA processing of a primary nuclear transcript containing the V_H , C_{μ} and C_{δ} genes. We have now studied the organization of C_H genes in sorted $\mu^+\epsilon^+$ B lymphocytes and found that they retain C_{μ} , C_{δ} , C_{γ} and C_{ϵ} genes, suggesting that the simultaneous expression of the C_{μ} and C_{ϵ} genes is mediated by an RNA splicing mechanism. We propose that class switching requires at least two steps of differentiation, the first step involving activation of differential splicing and the second the DNA rearrangement.

During experiments to define the new mouse C_H locus encoding IgE (*Igh-7*)¹⁷, we found that the *Igh*-congenic strain SJA/9 (*Igh^a*), which has a SJL background, cannot produce a detectable amount of IgE in the sera even after infection with *Nippostrongylus brasiliensis*, which is known to stimulate polyclonal IgE production. Furthermore, we found that the increase of IgE-bearing B cells after *N. brasiliensis* infection occurs equally in SJA/9 and SJL mice (K.O. et al., in preparation). Approximately 10% of the spleen cells of the *N. brasiliensis*-infected mice carry IgE on their surface, providing a unique opportunity for identifying sufficient IgE-bearing B lymphocytes for molecular genetic analyses. The advantage of SJA/9 mice is that a low IgE level in sera minimizes the binding of IgE to Fc receptors of ϵ -negative lymphocytes, thus avoiding the contamination of ϵ -negative cells into sorted ϵ^+ B cells. This strategy—taking advantage of a low level of a certain isotype in sera—was successfully used to purify IgG2a-bearing B cells from allotype-suppressed mice by fluorescence activated cell sorter¹⁸.

The IgE-bearing B cells were isolated from spleen cells of *N. brasiliensis*-infected SJA/9 mice using the fluorescence-activated cell sorter. Only the brightest top 9% of the stained cells were collected and their purity examined under the fluorescent microscope, which is less sensitive and gives a lower limiting value of staining. As shown in Table 1, at least 86% of the sorted cells were brightly stained with anti- ϵ antibody. Most of the ϵ -bearing cells also carried the μ chain on their surface.

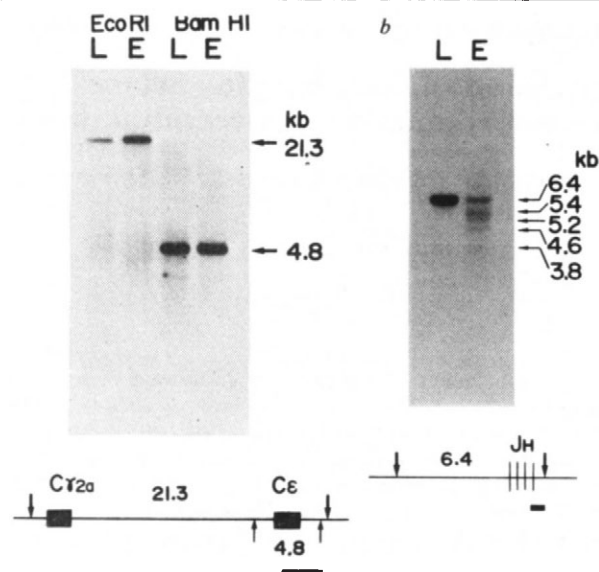


Fig. 1 Analysis of *Eco*RI and *Bam*HI fragments of ϵ -bearing cell and SJA/9 liver DNAs using cloned mouse C_{ϵ} and J_{ϵ} genes as probes. DNA was isolated from sorted ϵ^{+} B cells according to the method of S. Gattoni (personal communication). About 8×10^6 cells were lysed in 0.5 ml of 0.1% SDS, 10 mM EDTA 10 mM Tris-HCl pH 8.0, 10 mM NaCl, 200 μ g ml $^{-1}$ proteinase K for 90 min at 30°C with gentle shaking. An equal amount of neutralized heated (60°C) phenol was added to the lysate and tilted for 15 min. A few millilitres of ethanol were added to the water phase and DNA was wound up at the interphase with a glass rod. DNA was washed in a large amount of ethanol and dissolved in 100 μ l H $_2$ O. About 6 μ g DNA was obtained. Liver DNA was isolated as described before. The fragments obtained by *Eco*RI and *Bam*HI digestions of ϵ -bearing cell DNA (E) and SJA/9 liver DNA (L) were fractionated by electrophoresis in a 0.5% agarose gel, transferred to nitrocellulose filters and hybridized to the cloned mouse C_{ϵ} (a) and J_{ϵ} (b) probes which are indicated by wide bars below the restriction maps. In b, only *Eco*RI digests were analysed. Each lane contains $\sim 1 \mu$ g DNA. Probes were labelled by nick-translation and hybridized as described⁹. In the restriction maps closed rectangles indicate structural genes. Numbers show lengths (kb) of fragments. $\downarrow$, *Eco*RI; $\uparrow$, *Bam*HI.

However, they were not stained with anti- δ , anti- $\gamma 2a$ or anti- $\gamma 1$ antibody (data not shown). As a control, about 8% of the ϵ -depleted cells and 10% of the whole spleen cells were stained with anti- ϵ antibody. The results indicate that the sorted cells are the essentially pure population of $\mu^{+}\epsilon^{+}$ B cells.

To confirm that IgE on the surface of $\mu^{+}\epsilon^{+}$ B cells is endogenously synthesized, ϵ^{+} B cells were treated with trypsin (2.5 mg ml $^{-1}$) for 30 min at 37°C to strip off all cell-surface immunoglobulins, then after culturing, newly synthesized immunoglobulins on the surface were re-examined by fluorescence staining. As expected, 2 and 5 h after the trypsin treatment, 84 and 97%, respectively, of cells were stained with anti- ϵ . Furthermore, the sorted ϵ^{+} B cells of SJA/9 were shown to secrete IgE when T cells of SJL were provided (K.O. *et al.*, in preparation).

We have extracted DNA from the sorted $\mu^{+}\epsilon^{+}$ cells and examined the C_{ϵ} gene organization in ϵ -bearing cells using the Southern blotting technique. When DNA of ϵ^{+} B cells was digested with *Eco*RI, blotted and hybridized with the C_{ϵ} gene probe, it produced a 21.3-kilobase (kb) fragment identical to that produced in SJA/9 liver DNA (Fig. 1a). Similarly, *Bam*HI digestion of DNAs of ϵ^{+} B cells and SJA/9 liver yielded an identical fragment (4.8 kb) hybridizing with the C_{ϵ} probe. As the *Eco*RI fragment (21.3 kb) encompasses the whole region between the $C_{\gamma 2a}$ and C_{ϵ} genes, the above results indicate that the C_{ϵ} gene does not rearrange in the IgE-bearing lymphocytes, unlike the IgE-secreting hybridoma and myeloma^{19,20}.

On the other hand, the J_{ϵ} gene fragment of the IgE-bearing lymphocyte DNA drastically reduced the intensity as compared

Table 1 Characterization of sorted ϵ -bearing cells

Cells	Surface immunoglobulin-positive cells (%)	
	μ	ϵ
Whole spleen cells	46	9.6
Sorted cells		
Sorted ϵ^{+} B cells	77	86
The remaining cells	45	8.3

SJA/9 mice were infected with *N. brasiliensis* by subcutaneous injection of third-stage larvae (750 per mouse). The mice were killed 2 weeks later and their spleens gently teased to obtain lymphocytes. Spleen lymphocytes were stained with guinea pig anti-murine IgE sera¹⁷ and fluorescein isothiocyanate-labelled rabbit anti-guinea pig IgG1 antibodies. Stained spleen cells were sorted on the FACS III (Becton-Dickinson). An aliquot of the sorted cells and spleen cells were stained with biotin-conjugated rabbit anti-murine μ antibodies and rhodamine isothiocyanate-labelled avidin after photobleaching. As stained cells were counted under the fluorescent microscope, the values shown are lower limits.

with that of SJA/9 liver DNA, and appeared blurred, in agreement with the interpretation that a large number of different rearrangements have generated many new *Eco*RI fragments of different lengths in polyclonal B cells²¹ (Fig. 1b). Each of several faint bands (5.4, 5.2, 4.6 and 3.8 kb) having less than a few per cent of the intensity of the germ-line band in liver DNA, may represent rearranged J_{ϵ} genes for antibodies against *N. brasiliensis* antigens *per se*.

When DNA of IgE-bearing cells was digested with *Eco*RI, blotted and hybridized with the $C_{\gamma 2b}$ - $C_{\gamma 2a}$ probe, it produced the 6.6- and 21.3-kb fragments which correspond to the germ-line forms of the $C_{\gamma 2b}$ and $C_{\gamma 2a}$ genes, respectively (Fig. 2a). The germ-line $C_{\gamma 2b}$ and $C_{\gamma 2a}$ gene fragments (9 and 6.2 kb, respectively) were also detected in DNA of IgE-bearing cells when digested with *Hind*III. Similarly, *Eco*RI or *Hind*III digestion of IgE-bearing cell DNA produced the germ-line forms of the $C_{\gamma 1}$ and $C_{\gamma 3}$ genes (data not shown). In fact, the faint 23-kb *Hind*III fragment is the germ-line $C_{\gamma 1}$ gene detected by cross-hybridization of the $C_{\gamma 2b}$ probe (Fig. 2a).

*Eco*RI digestion of the ϵ^{+} B-cell DNA produced the germ-line form of the C_{μ} gene fragment (13 kb) (Fig. 2b). Inasmuch as the 13-kb C_{μ} fragment contains the whole S_{μ} region, there

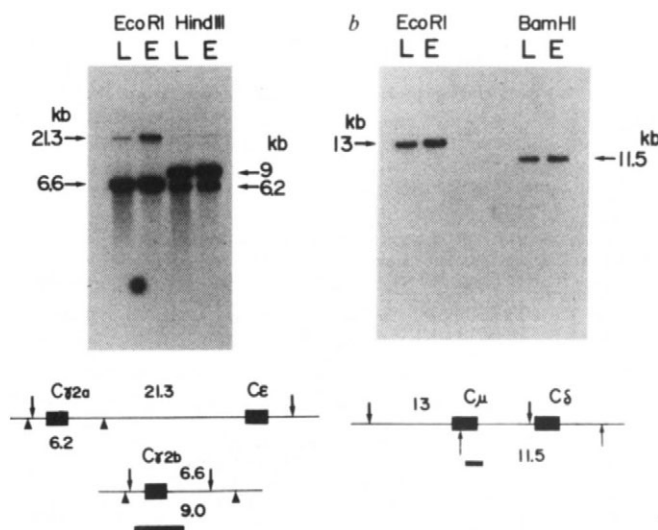


Fig. 2 Analysis of *Eco*RI, *Hind*III and *Bam*HI fragments of ϵ -bearing cell and SJA/9 liver DNAs using cloned mouse $C_{\gamma 2b}$ and C_{μ} genes as probes. Experimental conditions are as described in Fig. 1 legend. The fragments produced by *Eco*RI, *Hind*III and *Bam*HI digestion of ϵ -bearing cell DNA (E) and SJA/9 liver DNA (L) were electrophoresed and blotted to nitrocellulose filters. The restriction maps surrounding probes used [$C_{\gamma 2b}$ (a) and C_{μ} (b)] are shown below. Each lane contains $\sim 1 \mu$ g DNA. $\blacktriangle$, *Hind*III; $\downarrow$, *Eco*RI; $\uparrow$, *Bam*HI.

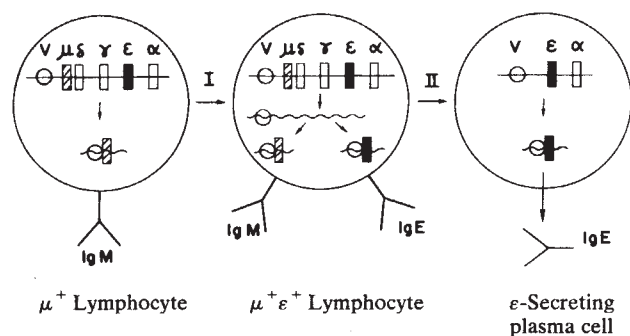


Fig. 3 Two steps of differentiation from μ^+ lymphocytes to ϵ -secreting plasma cells. Step I involves the activation of differential splicing. Alternate splicing of a long transcript containing V_H , C_μ , C_γ and C_ϵ sequences will produce mRNA encoding either μ or ϵ chain with the same V-region sequence. Step II involves DNA deletion. See the text.

is no doubt about the absence of the DNA rearrangement in the S_μ region. *Bam*HI digestion of the ϵ^+ B-cell and SJA/9 liver DNAs yielded the 11.5-kb fragment hybridizing with the C_μ probe. As the 11.5-kb *Bam*HI fragment encompasses the C_δ as well as the C_μ gene, the results indicate that the C_δ gene is not rearranged in the ϵ^+ B cells.

The intensity of each C_H gene band was quantified at the corresponding size by hybridizing the same filters used in the above experiments with the β -globin and ribosomal RNA gene probes. The ratio of the intensity of each C_H gene band to the β -globin and ribosomal RNA gene bands as determined by densitometer tracings remained constant regardless of the origin of DNA, that is, from liver or ϵ^+ B cells. These results suggest that the organization of the C_H gene in the IgE-bearing cells is probably the same as the germ-line gene except that the J_H is rearranged. These data indicate that IgE expression in $\mu^+ \epsilon^+$ B cells does not involve the S_μ - S_ϵ rearrangement although it is well established that the intervening DNA segment is deleted by the S-S recombination in various immunoglobulin-secreting B-cell lymphomas, hybridomas and myelomas including IgE secreters⁵⁻⁹.

Given these results we propose that differentiation of IgM-bearing B lymphocytes to IgE-secreting plasma cells may proceed by at least two biochemical steps, as shown in Fig. 3. The first step (step I) promotes differentiation of IgM-bearing B lymphocytes into IgM-IgE-bearing B lymphocytes, which involves the activation of differential RNA processing of a single large RNA transcript containing V_H , C_μ , C_δ , C_γ and C_ϵ gene sequences. The large transcript may be spliced into μ or ϵ mRNA by specific enzymes and/or specific assisting molecules such as low molecular weight RNA^{22,23}. The size of the primary transcript is estimated to be ~ 180 kb from the C_H gene organization¹. Naturally, the μ and ϵ mRNAs share an identical V_H region sequence. As we handled a mixed population of $\mu^+ \epsilon^+$ lymphocytes, we were unable to determine whether the same V_H sequence was associated with the C_μ and C_ϵ sequences in a single cell. However, IgD and IgM molecules were shown to bear the identical V_H region in a $\mu^+ \delta^+$ lymphoma¹⁶. We presume that step I does not involve any major DNA rearrangement.

IgM-IgE-bearing B lymphocytes differentiate into IgE-secreting B cells or plasma cells by step II, which involves S_μ - S_ϵ recombination and simultaneous DNA deletion as established previously²⁻⁹. Obviously, a similar mechanism should apply to the switch from IgM-bearing cells to IgD-, IgG- or IgA-secreting plasma cells. It is not clear whether DNA rearrangement accompanies the differentiation from IgM-bearing B cells to IgM-secreting plasma cells. Interestingly, most of the IgM-secreting myelomas and hybridomas seem to have a deletion in the S_μ region^{7,24,25}. We think it reasonable that deletion of the S_μ region facilitates the transcription of the C_μ gene and

promotes IgM secretion as the extremely G + C-rich S_μ region¹¹ may hinder efficient transcription.

There are no data concerning the length of the primary transcript of μ mRNA in IgM-bearing B cells. They may transcribe the whole C_H gene locus from the beginning. If so, step I is mediated by the activation of a new differential splicing system. Alternatively, the primary transcript in IgM-bearing B cells may contain only the V_H and C_μ sequences. In this case, step I requires at least two new biochemical events—the transcription of a much larger RNA and the activation of a new differential splicing system. To avoid the premature termination of transcription, lymphocytes may have to introduce some biochemical changes in the C_H gene locus such as demethylation²⁶. In fact, the C_δ gene is demethylated in $\mu^+ \delta^+$ hybridoma but not in μ^+ lymphoma²⁷.

This model favours the hypothesis that the splicing as well as recombination mechanism is class specific. Otherwise, the isotype expression in B cells should be transient and multiple (more than three isotypes per cell) until they become plasma cells. Several lines of evidence suggest that the expression of a certain V_H sequence is closely associated with a specific C_H isotype. CBA/N mice have genetic defects which make them incapable of producing anti-phosphorylcholine antibody of any classes other than IgE whereas anti-phosphorylcholine antibody of IgM and IgG is very common in most mouse strains^{28,29}. A lymphoma cell line I.29 has been shown consistently to switch from μ to α ³⁰. Such results seem to indicate that the S-S recombination is catalysed by the class-specific enzyme(s).

IgM-IgE-bearing lymphocytes accumulated in spleens of *N. brasiliensis*-infected SJA/9 mice are capable of differentiating into IgE-secreting plasma cells when T cells of SJL are provided (K.O. *et al.*, in preparation). As SJA/9 mice can synthesize normal amounts of IgM, IgG and IgA, the defect of SJA/9 seems to reside in IgE-specific regulatory T cells³¹. Furthermore, it is probably at step II that the T cells affect B-cell differentiation.

After completion of this manuscript we learned that Perlmutter and Gilbert³² had found the C_μ gene in γ_1 -bearing B cells purified from normal spleen using antibody-coated Petri dishes.

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Binding of four repressors to double-stranded *tet* operator region stabilizes it against thermal denaturation

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The investigation of protein-DNA interactions benefits from methods for the dissection of chromosomal DNA into handy fragments and their subsequent preparation in large amounts^{1,2}. Of particular interest are proteins active in gene regulation and their interaction with the control sequences of the respective genes³. Recently, we reported⁴ the purification of the molecular components of the control elements from the tetracycline-resistance (*tet*) gene located on the transposon Tn10. The Tet repressor inhibits transcription of the *tet* gene and its own gene⁴. When the *tet* operator was prepared on a 187 base pair (bp) DNA fragment⁴, the Tet repressor was found to bind specifically to this fragment with a stoichiometry of four Tet repressors per DNA fragment⁴. Tetracycline inhibits this binding⁴ and operates *in vivo* as an inducer for the expression of the Tn10-encoded tetracycline resistance^{5,6}. We now report thermal denaturation experiments of the Tet repressor-*tet* operator complex and demonstrate independently that four Tet repressor molecules bind to the 187 bp DNA and stabilize a 125 bp double-stranded DNA sequence against thermal denaturation.

Figure 1 summarizes the location of the biologically active sequences on the 187 bp DNA fragment. We investigated the thermal stability of the Tet repressor-*tet* operator complex by melting the 187 bp DNA in the presence of various amounts of Tet repressor. A stabilization can only be observed when the ionic conditions in the experiment are such that the thermal denaturation of the free DNA fragment occurs at a lower temperature than the denaturation of the complex. Previous results have suggested that the Tet repressor-*tet* operator complex is stable up to about 65 °C (ref. 4) at 0.2 M NaCl, 0.02 M MgCl₂. Assuming that the stability of the complex is limited by the thermal stability of the Tet repressor, then the melting experiments must be conducted at less than 40 mM NaCl because the *T_m* of the 187 bp fragment is 65 °C at that salt concentration. If, however, the report that non-

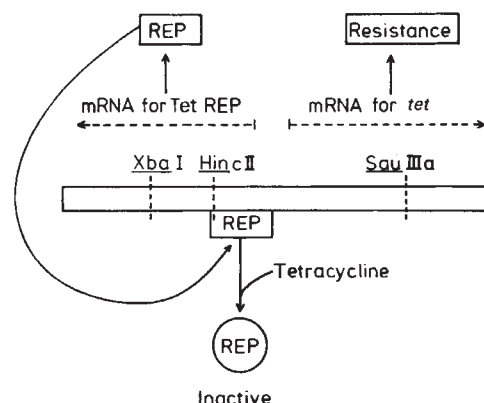


Fig. 1 Schematic presentation of the biologically active loci on the 187 bp *tet* operator DNA fragment (represented by the box). The recognition sites for the restriction endonuclease *Xba*I, *Hinc*II and *Sau*IIIa are indicated⁶. The *Hinc*II site is protected from cleavage by the presence of the Tet repressor (REP), whereas the *Xba*I and the *Sau*IIIa sites are not⁴. These results indicate the location of the *tet* operator region. The dashed lines represent the mRNAs for the tetracycline-resistance protein and the Tet repressor. The Tet repressor in the *tet* operator binding conformation is drawn as a box. It is inactivated in the presence of tetracycline as indicated by the circle⁴.

specific binding of the lac repressor to DNA increases with decreasing ionic strength^{7,8} were also to hold for the interaction of the Tet repressor with DNA, it would be difficult to distinguish nonspecific from specific binding. Figure 2 compares the effect of the Tet repressor on the thermal denaturation of two DNA fragments. Figure 2a shows the melting curves of the 95 bp DNA fragment containing the *Escherichia coli* lactose genetic control elements with (dashed line) and without (solid line) Tet repressor^{9,10}. This experiment serves as a control for nonspecific binding. The data reveal that the *T_m* of the 95 bp fragment is increased by 3.2 °C in the presence of the Tet repressor. This effect may be caused by either nonspecific binding of the Tet repressor to the DNA, or a slight increase in the ionic strength resulting from addition of the protein solution, or a combination of the two. As the ionic strength is only 6 mM in this experiment, even small alterations could cause this elevated *T_m*.

Figure 2b shows the dependence of thermal denaturation of the 187 bp *tet* operator fragment on Tet repressor concentration. In the absence of Tet repressor the 187 bp DNA melts in a single cooperative transition (solid line), whereas in the presence of a twofold molar excess of Tet repressor over 187 bp DNA, three melting transitions can be distinguished (dashed

Table 1 Melting temperatures and area analysis of the thermal denaturation experiments

Concentration of Tet repressor (M×10 ⁷)	Transition I		Transition II		Transition III		Ratio of area III:area II
	T _m (°C)	% Area	T _m (°C)	% Area	T _m (°C)	% Area	
187 bp <i>tet</i> operator DNA fragment							
0	56.8	100	—	—	—	—	—
1.1	58.3	70	59.5	12	65.3	18	1.5
2.4	59.3	36	60.5	27	66.3	37	1.4
3.6	60.8	18	61.8	33	67.5	49	1.5
4.7	—	—	62.0	39	67.5	61	1.6
9.3	—	—	61.8	39	67.3	61	1.6
95 bp <i>lac</i> operator DNA fragment							
0	62.4	100	—	—	—	—	—
6.3	65.6	100	—	—	—	—	—

The total concentration of the 187 bp DNA fragment was kept constant at $\sim 1.1 \times 10^{-7}$ M and the 95 bp DNA fragment at 1.6×10^{-7} M. Other experimental conditions were as indicated in Fig. 2 legend. The accuracy of *T_m* determination is approximately ± 0.3 °C as determined from repeat experiments. The total area was normalized to 100%. The accuracy of the area determination is approximately $\pm 8\%$ of the given value. This error arises mainly from the lack of distinction between peaks I and II (Fig. 2b). The number of base pairs in transition III was determined from melting experiments at 272 nm and is 125 ± 7 bp.

line). A van't Hoff analysis^{11,12} reveals that transition I arises from denaturation of the remaining free 187 bp fragment. Transitions II and III are the result of the thermal denaturation of the Tet repressor–187 bp DNA complex. The dissociation rate constant of the repressor–operator complex must be low, because it takes 100 min to go from transition I to transition III in this experiment (see Fig. 2 legend). In these conditions, the operator sequence is stabilized by $\sim 7^\circ\text{C}$ in the complex. In the presence of a fourfold molar excess of Tet repressor over 187 bp DNA (dotted line), all the 187 bp fragment melts as the protein–DNA complex in transitions II and III.

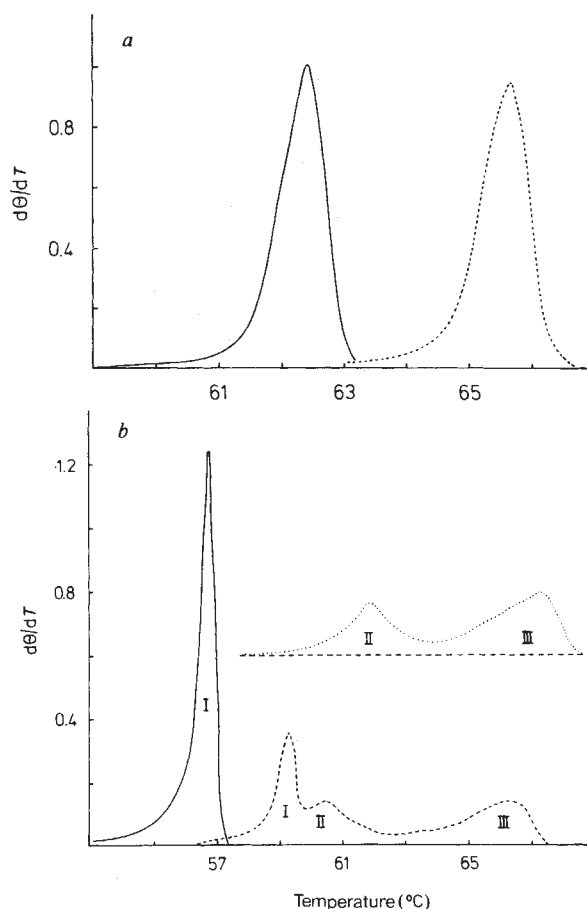
Table 1 summarizes the results from this experiment, carried out with a range of Tet repressor concentrations, together with the data from the 95 bp melting experiments. Three conclusions may be drawn. (1) Whereas the area of subtransition I decreases and those of peaks II and III increase with increasing Tet repressor concentration, the ratio of the areas under peaks II and III remains constant, indicating that peak II always results from the denaturation of 62 ± 7 bp and peak III from that of 125 ± 7 bp. For the conversion of peak areas to number of base pairs, the total area of the melting transitions of a given species is set to 100%. Each subtransition then arises from the denaturation of the respective percentage of the total number of base pairs¹². It may therefore be concluded that the same species of protein–DNA complex is denaturing at all Tet repressor concentrations studied, which excludes contributions from nonspecific binding. The shift of denatured base pairs from peak I to peaks II and III with increasing Tet repressor concentration is the result of the higher proportion of the Tet repressor–operator complex. (2) With increasing amounts of Tet repressor, the T_m values of the subtransitions are shifted to higher temperature. The increase in T_m of transition I is probably the result of increasing ionic strength due to the added protein

solution. As, at low Tet repressor concentrations, all Tet repressors bind specifically, nonspecific binding cannot account for the increased T_m . Transition III represents the stabilization resulting from specific binding of the Tet repressor to the *tet* operator region. This specific stabilization is strong enough to disrupt the cooperativity of the melting process. Unoccupied sequences melt under transition II. They are more stable than the free DNA melting in transition I because they are next to the stabilized repressor–operator complex. Therefore, the single strands cannot dissociate, and this accounts for the difference in T_m between transitions I and II. (3) The T_m of peak III does not increase over 67.5°C . This temperature must therefore be the denaturation temperature of the Tet repressor–*tet* operator complex. Thermal denaturation experiments with increasing ionic strength (data not shown) reveal that the stability of the complex is hardly affected by the salt concentration.

Based on this interpretation, the analysis of the melting curves in the presence of increasing amounts of Tet repressor should yield a binding curve for the Tet repressor–*tet* operator complex. If the fraction of DNA bound to the Tet repressor is calculated from an area analysis of peaks II and III with the areas of peak I representing the fraction of free DNA, the binding curve displayed in Fig. 3 is derived. The result indicates a stoichiometry of 3.6 Tet repressor molecules per DNA fragment, which, considering the uncertainties involved in determining the active fraction in a given protein preparation, may be interpreted as a 4:1 stoichiometry in the Tet repressor–*tet* operator complex.

In the *E. coli* lactose genetic control region, the stability of the DNA is correlated to the genetic functions of the region^{9,10}. The binding sites for cyclic AMP receptor protein and RNA polymerase are located in one cooperatively melting 80 bp region of the DNA, on the boundary of which is the lac repressor

Fig. 2 Thermal denaturation of the 95 bp *lac* operator and the 187 bp *tet* operator DNA fragments in the presence of various amounts of Tet repressor. *a*, Melting transition of the 95 bp *lac* operator DNA fragment alone (—) and in the presence of a fourfold excess of Tet repressor (-----). The only effect of the Tet repressor is a shift of the T_m from 62.4 to 65.6°C . In both experiments, the concentrations of the 95 bp fragment and the Tet repressor were 1.6×10^{-7} M and 6.3×10^{-7} M respectively. The shift of T_m is interpreted as a nonspecific effect arising from addition of the protein solution. *b*, Melting transitions of the 187 bp *tet* operator DNA fragment in the presence of various amounts of Tet repressor. The 187 bp DNA alone (—) melts in a single cooperative transition designated I. The concentration of the 187 bp fragment in all experiments was 1.1×10^{-7} M. In the presence of 2.4×10^{-7} M Tet repressor, three melting transitions are detectable (-----). Transition I results from the remaining free 187 bp fragment. Nonspecific effects probably arising from salts present in the Tet repressor solution shift the T_m by 2.5°C . Results from the experiment in the presence of 4.7×10^{-7} M Tet repressor (.....) suggest that transitions II and III arise from the denaturation of the Tet repressor–*tet* operator complex. This melting curve is offset by 0.6 on the vertical axis. In these conditions, no free 187 bp DNA is left and the entire denaturation occurs under peaks II and III. The amount of active Tet repressor in the protein preparation used for this experiment was determined by titration with ^3H tetracycline assuming a stoichiometry of 1:1 for the Tet repressor–tetracycline complex as described previously⁴. The salt conditions in the thermal denaturation experiments were 3.5 mM NaCl, 5 mM sodium cacodylate, pH 7.0, and 0.1 mM EDTA. Electrophoresis of the samples on 5% acrylamide gels before and after the thermal denaturation experiments confirmed the absence of any degradative activity during the measurements. The thermal denaturation was measured using a Zeiss PMQ III spectrophotometer at 260 nm. The cuvette was heated by a Haake thermal bath equipped with a Haake PG 10 temperature controller. The heating rate was 0.07°C per min. The temperature was monitored by placing a NTC resistor in the circulating liquid behind the cuvette. The Zeiss and the NTC were interfaced to a Commodore computer model 3016. Between 180 and 250 temperature–absorbance data pairs were recorded per experiment. The data were smoothed by fitting segments of 13–21 data pairs to a linear equation. Less rigorous smoothing procedures ensured the absence of any effect of the smoothing procedure on the melting curve. The total hypochromicity was normalized to 1 and the melting curve was differentiated to produce the figures.



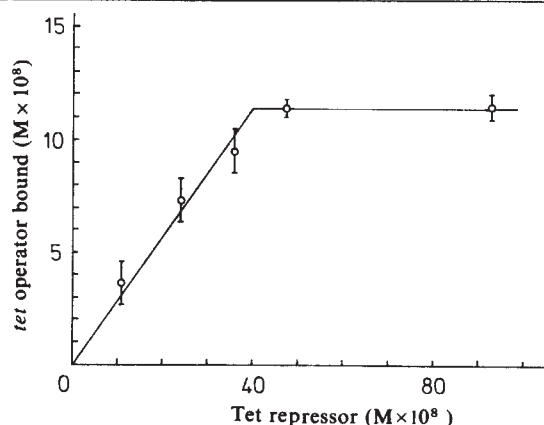


Fig. 3 Binding curve of the 187 bp *tet* operator DNA fragment to the Tet repressor. The areas of peak I (Table 1) for different Tet repressor concentrations were analysed to yield the concentration of bound 187 bp DNA, which was plotted versus the total Tet repressor concentration. The resulting binding curve indicates quantitative binding in these conditions. The stoichiometry obtained from this curve is ~3.6 Tet repressors per 187 bp fragment.

binding site^{9,10}. A similar arrangement has been reported for the *tet* gene regulatory region on Tn10, where the operators and promoters are located on a 140 bp cooperatively melting part of the DNA¹³. Our demonstration here of the specific stabilization of this operator-promoter sequence by the Tet repressor suggests that transcription is regulated by a change in the stability of the double strand. In this view, a repressor would be expected to increase the stability of the double strand, which we have indeed shown to be the case, whereas an activator would have the opposite effect.

The data presented here suggest that the Tet repressor recognizes a double-stranded conformation of the *tet* operator which is considerably stabilized in the complex. A possible cruciform structure of the *tet* operator would involve single-stranded regions of the DNA, but such regions could not serve as a Tet repressor binding site because a lower T_m would be predicted for a single-stranded binding protein complexed to DNA¹⁴. The thermal denaturation of the Tet repressor-*tet* operator complex serves as a new method to monitor specific complex formation because the free and bound DNA is clearly distinct and the dissociation rate constant is low enough for the fraction of each to be determined⁷. The constant stoichiometry during the titration implies strong cooperativity of complex formation. Cooperativity in repressor-operator binding has also been observed for the λ repressor¹⁵. Because there may be two operator regions on the 187 bp fragment⁶, it remains to be shown whether this cooperativity is the result of protein-protein contacts or of a conformational change in the DNA on binding the first Tet repressor.

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Spherical *E. coli* due to elevated levels of D-alanine carboxypeptidase

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The characteristic shape of bacterial cells is maintained by the strength and form of the murein layer of the cell wall¹. Thus, rod-shaped bacteria need to synthesize a rod-shaped murein. The shape of the murein may be determined by the properties of the penicillin-binding proteins (PBPs) that catalyse the insertion of murein precursors into the cell wall². In *Escherichia coli* inhibition of PBPs produces characteristic effects on bacterial morphology. For example, inactivation of PBP 2 results in an inability to synthesize cylindrical murein during cell elongation and the growth of *E. coli* as spherical cells³. We report here that osmotically stable spherical cells of *E. coli* can also be produced by an increase in the level of PBP 5, a D-alanine carboxypeptidase⁴, and show that these cells, and those produced by inactivation of PBP 2, have the same abnormality in the structure of the newly inserted murein.

PBP 5 of *E. coli* is one of the major D-alanine carboxypeptidases that remove the terminal D-alanine from the pentapeptide side chains of the murein^{2,3}. The PBP 5 gene (*dacA*) is carried by *ldl*p5⁴. DNA from *ldl*p5 was digested partially with *Sau*3A and fragments of 1-5 kilobases (kb) were purified and inserted into the *Bam*HI site in the tetracycline resistance gene of the low copy number plasmid pSC105⁴. Plasmids carrying *dacA* were detected by the reappearance of PBP 5 in an *E. coli* strain deleted of the chromosomal *dacA* gene⁶. pBS25, which has a 3-kb chromosomal insert, was chosen for further study, and pBS32, which is pSC105 with a deletion of the tetracycline resistance gene, was used as a control plasmid. Figure 1 shows the elevated level of PBP 5 in C600 (pBS25) compared with C600 (pBS32) control cells.

E. coli C600 (pBS25) grew as spherical cells in all media tested (Fig. 2) and plated with low efficiency on nutrient agar but plated normally on minimal media. To establish that the spherical growth of *E. coli* was a direct result of the elevated level of PBP 5, rather than of the product of some other gene on pBS25, we subcloned the *dacA* gene on a 1.5-kb *Eco*RI-*Bam*HI fragment into a derivative⁷ of pSC105. *E. coli* C600 carrying the resulting plasmid (pBS42) also grew as spherical cells. Insertion of DNA fragments into unique *Bst*EII, *Sal*I and *Bgl*II sites within the 1.5-kb *dacA* fragment inactivated *dacA* and in each case these plasmids had no effect on cell morphology. Similarly, a series of deletions extending from the *Eco*RI or the *Bam*HI ends of the *dacA* fragment in pBS42 were constructed, and in all cases plasmids with deletions extending into the *dacA* gene had no effect on cell morphology, whereas deletions that left *dacA* intact produced a spherical morphology. Spherical cells therefore result from the elevated levels of the *dacA* gene product, PBP 5.

E. coli cells that are rod-shaped at 30°C and become spherical at 42°C have been obtained by placing the expression of the *dacA* gene under the control of the phage λ leftward promoter and a thermolabile λ repressor, in a low copy number plasmid expression vector⁷. This strain eventually dies at 42°C, presumably because the high levels of PBP 5 remove all the

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Table 1 Murein metabolism in spherical cells produced by elevated levels of PBP 5

Strain	New murein	Cross-linkage	Old murein	Pentapeptide in new murein (%)	D-alanine carboxypeptidase activity (U)
C600(pBS32)	25.3 ± 1.3		32.4 ± 1.9	2.5	10.1
C600(pBS25)	30.2 ± 1.7		32.8 ± 1.7	1.5	37.8

Cross-linking in old murein: cells were grown at 37 °C for four generations in minimal medium containing kanamycin (50 µg ml⁻¹) and ³H-diaminopimelic acid (2.5 µCi ml⁻¹; 50 Ci mmol⁻¹) to an absorbance at 578 nm of 0.6. Cross-linkage in new murein: cells were grown at 37 °C in minimal medium containing kanamycin (50 µg ml⁻¹) to an absorbance at 578 nm of 0.6 and samples (4 ml) were labelled with ³H-diaminopimelic acid (30 µCi ml⁻¹) for 4 min. The labelled cells were processed as described previously⁸, except that digestion with lysozyme was preceded by a 6-h incubation of the murein with amylase (100 µg ml⁻¹), and the level of the cross-linkage was determined as described previously⁸. Values given are the average of eight separate experiments. The pentapeptide content of the newly inserted urein was determined as described elsewhere⁸. In each of four independent experiments the pentapeptide level in C600(pBS25) was 48–73% of that in C600(pBS32), although the variation between experiments was rather large. Crude extracts from sonicated cells suspended in 0.5 M Tris-HCl buffer (pH 8.6) containing 2% Triton X-100 were used to determine carboxypeptidase activity which was assayed by measuring the release of ¹⁴C-D-alanine from UDP-N-acetylmuramyl pentapeptide labelled in the terminal D-alanine (specific activity 21 c.p.m. per pmol). The reaction mixture contained 0.5 nmol labelled substrate, 0.1 M Tris-HCl buffer (pH 8.6), 0.4% Triton X-100 and extract in a volume of 25 µl. The reaction was terminated by boiling and product and substrate were separated by ascending paper chromatography (Whatman 3MM paper) in a solvent system of isobutyric acid: 1 M ammonia (5:3; vol/vol). One unit of carboxypeptidase is defined as that amount of enzyme that will release 1 pmol of ¹⁴C-D-alanine from the substrate in 30 min at 37 °C.

terminal D-alanine residues from murein precursors and prevent further murein synthesis. Probably for the same reason *dacA* cannot be cloned into high copy number plasmid vectors.

The level of PBP 5 in C600 (pBS25) was about 10 times that in control cells. The level of total D-alanine carboxypeptidase in C600 (pBS25), which is due to PBPs 4, 5 and 6 (ref. 2), was 3.7 times higher than in control cells (Table 1). The elevated level of PBP 5 resulted in a decreased amount of pentapeptide side chains in the newly inserted murein (Table 1), supporting the view that PBP 5 acts as a D-alanine carboxypeptidase *in vivo*⁸.

Cross-linking of the murein of *E. coli* occurs in two stages⁹. Initial insertion of murein precursors by transpeptidation, catalysed by high molecular weight PBPs^{2,10,11}, results in a low level of cross-linking in the newly inserted murein. Subsequently, further cross-linking of the inserted murein occurs to produce the level of cross-linking characteristic of the mature wall. As expected, the level of cross-linking in the newly inserted murein of control cells of C600 (pBS32) was lower than in old murein (Table 1). The level of cross-linking in the old murein of the spherical cells of C600 (pBS25) was the same as in the control cells, but the level in newly inserted murein was abnormally high, and was almost the same as in the old murein (Table 1).

The abnormal level of cross-linkage in the newly synthesized murein of the spherical cells of C600 (pBS25) was also found in *E. coli* K-12 cells made spherical by inactivation of PBP 2. In three separate experiments the newly synthesized murein was ~20% more cross-linked after growth in the presence of

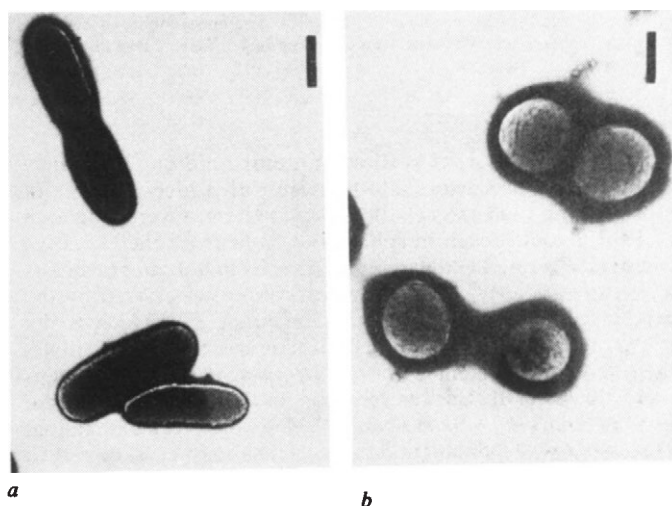


Fig. 2 Morphology of *E. coli* C600 (pBS25). Electron micrographs of negatively stained C600 (pBS32) (a); and C600 (pBS25) (b). Cells were grown at 37 °C in minimal medium containing kanamycin (50 µg ml⁻¹). Scale bars, 1 µm.

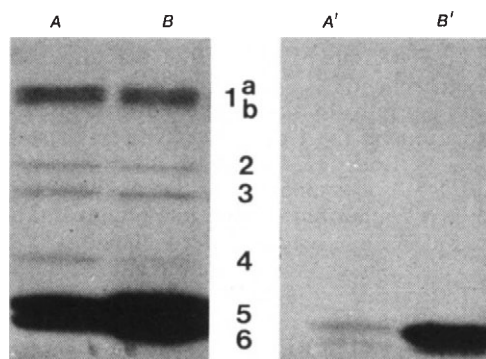


Fig. 1 Overproduction of PBP 5 in *E. coli* C600 (pBS25). C600 (pBS32) (A and A') and C600 (pBS25) (B and B') were grown to late exponential phase in minimal medium containing kanamycin (50 µg ml⁻¹) at 37 °C and cell envelopes were prepared and PBPs assayed as described previously⁴. The left and right halves of the figure show the same gel autoradiographed for 30 days and 3 days, respectively.

meccillinam (a specific inhibitor of PBP 2; ref. 12) than in control cells. The effect on cross-linking occurred within a few minutes of the addition of meccillinam and well before any shape change could be seen (Fig. 3). Similar results were obtained using the temperature-sensitive mutant, SP137, which becomes spherical at 42 °C due to inactivation of a thermolabile PBP 2 (ref. 12). The cross-linking in new murein was 30% higher in SP137 cells shifted to 42 °C for 40 min than in rod-shaped cells of SP137 maintained at 30 °C.

These experiments demonstrate that the production of spherical cells of *E. coli* by two completely different methods results in the same abnormality of murein synthesis. How does inactivation of PBP 2, or overproduction of PBP 5, result in a higher level of cross-linking in newly inserted murein and growth as spherical cells? It is unlikely that PBP 2 acts directly by modulating expression of PBP 5, or vice versa, as no changes in PBP 2 activity have been found in cells with elevated PBP 5 and no changes in PBP 5 activity accompany inactivation of PBP 2.

One possibility is that PBP 2 is a component of the enzyme system that incorporates new murein for cell elongation, and that in its absence murein synthesis occurs entirely via the system responsible for the synthesis of hemispherical murein during cell division. The latter system involves PBP 3, which

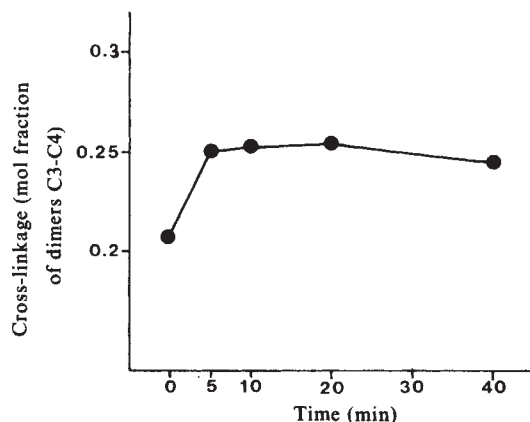


Fig. 3 Effect of mecillinam on cross-linking of newly inserted murein. *E. coli* PA3092⁸ was grown in minimal medium at 37 °C to an absorbance at 578 nm of 0.4 and was pulse-labelled with ³H-diaminopimelic acid (25 µCi ml⁻¹) for 4 min, before addition of mecillinam, or at various times after the addition of mecillinam (1 µg ml⁻¹). The cross-linking in newly inserted murein was measured as described in Table 1 legend.

seems to be a murein synthetase-transpeptidase¹³, and may insert murein precursors into the wall with a higher degree of cross-linking than the cell elongation system. Overproduction of PBP 5 could result in spherical cells because the excessive removal of terminal D-alanine residues from murein precursors may convert them to a form which cannot be utilized by the murein system involved in cell elongation, but which is the preferred substrate for the cell division system. For example, the cell division system might require precursors with tetrapeptide side chains that act as acceptors in the cross-linking reaction, as occurs in *Gaffkya homari*¹⁴, whereas, in cell elongation, precursors with pentapeptide side chains may be required to act as peptide donors in cross-linking. Increased conversion of pentapeptide side chains to tetrapeptide side chains in murein precursors would stimulate cross-linking by the cell division system, and suppress cross-linking by the cell elongation system, resulting in spherical growth.

Interestingly, the suggestion that D-alanine carboxypeptidase levels may be implicated in the switching between cell elongation and cell division has been proposed from completely different experiments that showed a high level of carboxypeptidase immediately before cell division and a low level in cells that elongate without dividing^{15,16}.

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In vitro packaging of plasmid DNAs into ΦX174 bacteriophage capsid

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Previously we have developed an *in vitro* system that synthesizes infectious single-stranded (ss) DNA bacteriophage ΦX174 from purified phage components¹. This system provides an assay to examine the function of each viral component in the phage synthesizing reaction. In this report, we show that the specificity of the template DNA for the phage synthesizing reaction is determined by the specific region of ΦX174 genome. Plasmid DNAs carrying the origin of ss DNA synthesis of ΦX174 are active as templates in the *in vitro* system and produce particles containing ss plasmid DNA packaged into ΦX174 capsids. The orientation of the ΦX174 DNA fragment carrying the origin determines which strand of the plasmid is packaged. The particles synthesized *in vitro* are infectious to ΦX174-sensitive *Escherichia coli* and introduce ss plasmid DNA into the host cells. The ss DNA is converted to ds plasmid which is maintained in the cell.

In *E. coli* cells infected with ΦX174, ss DNA is first converted to double-stranded (ds) replicative form (RF) DNA, which is then multiplied by semiconservative replication². During the late stage of infection, asymmetric ss DNA synthesis occurs^{2,3}. This DNA synthesis is coupled to the encapsidation of ss DNA into preformed proheads to form infectious phage particles. Both semiconservative replication of RF DNA and asymmetric ss DNA synthesis are initiated by the nicking action of ΦX174 gene A protein at a specific site (A site) on the viral strand of ΦX174 RFI DNA^{4,5}. The A site also acts as a signal for terminating these DNA syntheses⁶. However, it is not known whether the remainder of the ΦX174 sequence has some role in ss DNA synthesis and packaging of the ss DNA into prohead. This problem has been approached by cloning the A-site sequence from ΦX174 RF DNA into plasmid pACYC 184 (ref. 7) thereby isolating it from other ΦX174 DNA sequence and testing its template activity in the *in vitro* phage synthesizing system.

The third largest fragment of restriction endonuclease *HincII*-digested ΦX174 RF DNA (4,200 to 4,812 of the Sanger et al.'s map)⁸, which contains the A-site sequence, was inserted at the *EcoRI* site of plasmid pACYC184 using *EcoRI* linkers. Plasmid DNAs containing the fragment in either direction were isolated (pH24R and pH24L). Plasmid pH13, which contains fragment III from a restriction endonuclease *HaeIII* digest of ΦX174 RF DNA (4,948 to 434 of the Sanger et al.'s map) inserted at the *EcoRI* site of pACYC184, was also isolated for the control experiment.

The *in vitro* phage synthesizing system described previously¹ was used to examine the template activity of these plasmid DNAs (Table 1). Both pH24R and pH24L were as active as ΦX174 RFI DNA in supporting ss DNA synthesis and in packaging newly synthesized ss DNA into proheads. The particles synthesized in these reactions were infectious to ΦX174-sensitive *E. coli* as judged by the transduction of tetracycline resistance (Tc^r) carried by pH24R and pH24L. The synthesis of ss DNA and infectious particles required the same components as for the ΦX174 RFI DNA-directed phage synthesis, that is, template DNA, purified ΦX174 gene A protein, gene C protein, gene J protein, prohead and uninfected host cell extract. Neither pH13 DNA nor pACYC184 DNA could support synthesis or packaging of ss DNA, indicating that the cloned ΦX174 fragment containing the A-site sequence carried necessary and sufficient information for ss DNA synthesis and packaging of the ss DNA into particles.

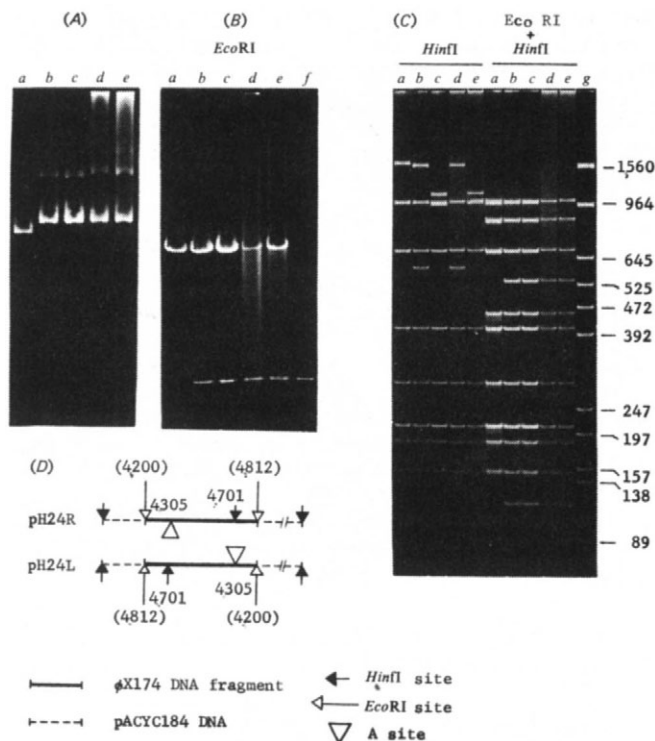


Fig. 1 Analyses of transduced DNA by gel electrophoresis. The *in vitro* reaction and the infection of *E. coli* HF4704 with the product were performed as described in Table 1 using either pH24R or pH24L DNA as template. DNA was isolated from Tc-resistant cells by the method of Birnboim and Doly⁹. DNA samples examined are: a, pACYC184; b, pH24R; c, pH24L; d, DNA extracted from Tc-resistant cells infected with *in vitro* particles synthesized in the pH24R DNA-directed reaction; e, DNA extracted from Tc-resistant cells infected with *in vitro* particles synthesized in the pH24L DNA-directed reaction. A, approximately 0.1 µg of each DNA was electrophoresed in a 1% agarose slab gel in Tris-borate buffer¹² (10.8g trisma base, 0.93g EDTA and 5.5g boric acid per litre) at 25 mA for 2 h. B, approximately 0.2 µg of each DNA was digested with endonuclease *EcoRI* and analysed as described in A. Fragment III of *HincII* digest of ΦX174 RF DNA which contains the A-site was electrophoresed as a marker (panel f). C, approximately 1.5 µg of each DNA was digested with endonuclease *HincII* or 4 µg of each DNA with endonucleases *HincII* and *EcoRI*. Gel electrophoresis was performed in a 7% polyacrylamide (acrylamide:methylene bisacrylamide = 29.2:0.8) slab gel containing 5% (v/v) glycerol in Tris-borate buffer at 10 mA for 16.5 h. ΦX174 RF DNA digested with endonuclease *RsaI* was electrophoresed as markers for molecular weight (panel g) and the number of base-pair of each fragment is shown. D, Schematic representation of the structure of pH24R and pH24L DNA. Each number shown is the position of Sanger *et al.*'s DNA sequence of ΦX174 genome. *HincII* sites of ΦX174 RF DNA (4,200 and 4,812 of the Sanger *et al.*'s map) were attached to *EcoRI* linkers and is represented as *EcoRI* sites. Gels were photographed under UV light after ethidium bromide staining.

DNA in the particles synthesized *in vitro* directed by pH24R and pH24L (pH24R* and pH24L*) were complementary as judged by the hybridization experiments (Table 2). Thus the orientation of the A-site sequence in the plasmid determines which strand of the plasmid DNA is synthesized and packaged into particles. Figure 1A shows that plasmid DNA isolated from Tc^r cells after infection with the *in vitro* particles (transduced DNA) co-migrated with the template DNA upon agarose gel electrophoresis. Fragments produced by endonuclease *EcoRI* (Fig. 1B) and *HincII* (Fig. 1C) digestion of transduced DNA also comigrated with their respective template DNAs. The *HincII* digest also shows that the template DNAs (pH24R and pH24L) have the A-site fragment inserted in opposite

Table 1 Template specificity and requirement for *in vitro* phage synthesizing system

Template DNA	Omission	ss DNA synthesis	Infectivity
ΦX174 RFI	None	148	7×10^{10}
pH24R	None	98	5×10^{10}
	A protein	5	6×10^8
	C protein	2	1×10^8
	J protein	7	$<10^3$
	Prohead	<1	$<10^3$
	Host protein fraction	<1	7×10^4
pH24L	None	90	8×10^9
	A protein	6	3×10^8
	C protein	4	4×10^7
	J protein	18	$<10^3$
	Prohead	<1	$<10^3$
	Host protein fraction	<1	3×10^4
pH13	None	<1	$<10^3$
pACYC184	None	<1	$<10^3$
None	None	<1	$<10^3$

Purified ΦX174 gene A protein, ΦX174 gene C protein, ΦX174 gene J protein, ΦX174 prohead, ΦX174 RFI DNA and *E. coli* protein fraction were prepared as previously described¹. Plasmid DNAs used as templates were isolated by the method of Birnboim and Doly⁹ and further purified by methylated albumin column chromatography¹⁰. *In vitro* reactions using 1 pmole of ΦX174 RFI DNA (molecular weight 3.4×10^6) or 1 pmole of plasmid pH24R (3.04×10^6 daltons), pH24L (3.04×10^6), pH13 (3.20×10^6), constructed *in vitro* as described in the text, or pACYC184 (2.65×10^6) were performed in the phage-synthesizing reaction mixture (25 µl) as described at 30°C for 30 min. The ss DNA synthesis is represented as pmole of deoxyribonucleotides incorporated into acid-insoluble, DNase-resistant materials in a 25-µl reaction mixture. DNase-resistant DNA is the DNA that has been packaged into proheads¹. The infectivity of the product directed by ΦX174 RFI DNA as template was determined by plaque assay using *E. coli* HF4704 (sup⁺) as indicator bacteria and is represented as plaque-forming units (PFU) per ml of reaction mixture. The infectivity of the products directed by plasmid DNA was determined by infecting HF4704 with dilutions of the reaction mixtures and spreading on LB-agar plates (10g tryptone, 5g yeast extract, 10g NaCl and 15g agar per l) containing 20 µg ml⁻¹ tetracycline. The infectivity is represented as the number of Tc-resistant cells produced per ml of reaction mixture.

Table 2 Hybridization experiment of *in vitro* packaged DNA

DNA source	c.p.m. hybridized
pH24R* + pH24R*	185
pH24R* + pH24L*	2,322
pH24L* + pH24L*	180

In vitro phage synthesizing reactions directed by either pH24R or pH24L DNA were performed as described in the legend to Table 1 except that the volume of the reaction mixture was 250 µl. After 30 min of reaction, DNase I was added to a final concentration of 0.1 mg ml⁻¹ and the mixture was incubated for another 30 min. Pronase and sodium dodecylsulphate were then added to final concentrations of 0.5 mg ml⁻¹ and 0.1% respectively and the mixture was incubated at 37°C for 3 h. DNA was extracted by the hot-phenol procedure¹¹. Each DNA sample (3,250 c.p.m.) was mixed and the mixture was adjusted to 35% formamide and 0.2 M sodium acetate (final volume 40 µl). After incubating at 35°C for 13 h, 160 µl of water and 20 µl of S₁ nuclease solution (40,000 units ml⁻¹ single-stranded specific nuclease S₁, 30 mM sodium acetate, pH 4.6 and 5 mM ZnCl₂) were added and the mixture was incubated at 37°C for 1 h. Ice-cold trichloroacetic acid (7%)-precipitable counts were taken as hybridized counts. pH24R*, DNA extracted from particles synthesized in pH24R DNA-directed reaction; pH24L*, DNA extracted from particles synthesized in pH24L DNA-directed reaction.

orientation (Fig. 1D). The pattern produced by digestion with *HincII* and *EcoRI* shows that the A-site sequence is inserted at the *EcoRI* site of pACYC184 (Fig. 1C, D). The recovery of the transduced DNAs as ds plasmid DNA indicates that the ss

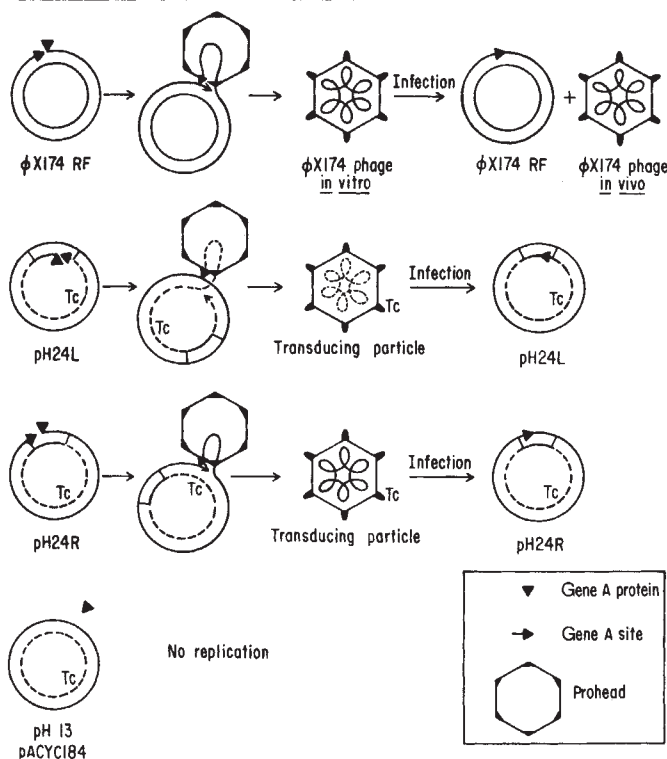


Fig. 2 Schematic representation of synthesis of infectious transducing phage *in vitro*. In the phage synthesizing system *in vitro*¹, ΦX174 gene A protein cleaves the viral strand of ΦX174 RFI DNA at its origin of replication (A-site) to form open circular RF (RFII) DNA whose 5'-terminus is covalently attached to gene A protein^{4,5}. The RFII DNA-gene A protein complex then associates with prohead and ss DNA synthesis occurs by a looped rolling-circle mechanism⁴. Host proteins and gene C, gene J proteins of ΦX174 are required in these processes¹ (not shown in figures). The displaced viral strand is encapsidated into prohead while DNA synthesis is occurring. When one round of DNA synthesis is completed, gene A protein cleaves the viral strand and joins the two ends to form a phage particle containing a circular viral strand⁶. Phage particles synthesized *in vitro* are infectious to ΦX174-sensitive *E. coli* cells. After infection, the circular ss DNA is converted to ds RF DNA, which is multiplied to produce progeny RF molecules. In the late stage of infection, progeny RF molecules serve as template for synthesis and packaging of ss DNA to produce phage particles by a similar mechanism as *in vitro* reaction. In the pH24R or pH24L DNA-directed *in vitro* reaction, ΦX174 gene A protein cleaves either one of the strands of the plasmid DNA at the A-site and forms a plasmid DNA-gene A protein complex. After associating with prohead, asymmetric ss DNA synthesis occurs as in the case of the ΦX174 DNA-directed reaction. Depending on the orientation of the A-site fragment, one of the strands of plasmid DNA is specifically displaced and encapsidated into prohead. Thus pH24R or pH24L DNA-directed reaction produces infectious particles in which complementary circular plasmid ss DNAs are packaged. After infecting ΦX174-sensitive *E. coli* cells, the plasmid ss DNA is converted to ds plasmid DNA, which is multiplied and maintained in the cell. As a result, tetracycline-resistance carried by pH24R and pH24L is transformed to *E. coli* cells. Neither pH13 DNA nor pACYC184 DNA support synthesis or packaging of DNA due to the lack of the A-site sequence in these plasmid DNAs.

DNA in the transducing particle is converted to and maintained as the ds form in the cell. The maintenance of the transduced plasmid probably occurs via the plasmid replication pathway because it depended on the presence of polA1 protein (data not shown).

The synthesis and packaging of ss DNA from pH24R and pH24L to produce transducing particles also occurred *in vivo* when cells harbouring these plasmids are infected with wild type ΦX174. The infecting phage apparently provides the

necessary phage-originated proteins to produce the transducing particles by a mechanism similar to that occurring in the *in vitro* system described in this report. A schematic representation of the *in vitro* reactions is shown in Fig. 2.

This system will be useful in elucidating the biochemical functions of gene C and gene J proteins. By varying the size of the plasmid carrying the A-site sequence, this system also provides an excellent way to determine the minimal and maximal lengths of ss DNA able to be packaged into the prohead structure—an important parameter for the analysis of phage morphogenesis. The system described in this report also provides some applications in the molecular cloning of DNA. The efficiency of infection of particles containing plasmid DNA (as measured by the transduction of the drug marker) is as high as the efficiency of plating of phage, thereby providing several magnitudes higher transfer efficiency of plasmid DNA into cells over the conventional transformation method. The strand separation of plasmid DNA resulting from its packaging may also be useful in DNA sequencing.

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Errata

In the letter 'A 2-D model calculation of atmospheric lifetimes for N₂O, CFC-11 and CFC-12' by M. K. W. Ko and N. D. Sze, *Nature* **297**, 317–319 (1982), figures 2 and 3 have been transposed.

In the letter 'Helium isotopic systematics of oceanic islands and mantle heterogeneity' by M. D. Kurz *et al. Nature* **279**, 43–47 (1982), on page 45 the first two lines (with age ... degassed of ³He) should be transposed to the bottom of the column.

Corrigenda

In the letter 'Structural alterations in J regions of mouse immunoglobulin λ genes are associated with differential gene expression' by J. Miller, E. Selsing and U. Storb, *Nature* **295**, 428–430 (1982), the sequence of the dJλ2 recognition nanomer is incorrect as shown in Fig. 2. The sequence should read GGATCTTGC. This change (underlined) only results in a closer resemblance of the dJλ2 recognition sequence to the consensus, making dJλ2 more like ψJλ3 and, therefore, more likely to interfere with functional Vλ2–Cλ2 joining. Thus, the error does not affect the conclusions presented.

In the letter 'Electrophysiology of mammalian thalamic neurones *in vitro*' by R. Llinas and H. Jahnsen, *Nature* **297**, 406–408 (1982), in line 4 in the right-hand column on page 407 the term 'early Na conductance' should read 'early K conductance'.

BOOK REVIEWS

Porcupine biology

A. J. Cain

THIS solid book (almost 900 pages in length) is essential reading for everyone at all interested in evolution, in biology or its history, or in science in general. Ernst Mayr is of course a contributor to the modern theory of evolution of top international standing, which instantly gives him a status far above most of those who have written on the history of biology; but also, he is a member by birth of one great European culture and a participant by adoption in the vast North American ethos. With such insights as are given by multilingual ability, breadth of culture, and a great practical experience and successful achievement in evolutionary research, one might expect him to produce a far more balanced, penetrating and illuminating book than could most others. Taking the book as a whole, it comes well up to expectation.

Mayr is as concerned with the conceptual structure of biology and its place among the sciences as with the development of particular themes within it. The introduction on how to write the history of biology, and the following chapter, "The Place of Biology in the Sciences and Its Conceptual Structure", develop his general methodology and viewpoint on the nature of biology; and the epilogue, "Towards a Science of Science", sums up and justifies much of his approach in the light of the findings in the main part. This latter falls into three parts: "Diversity of Life", "Evolution" and "Variation and Its Inheritance". Each of these is a fascinating history, from earliest times virtually up to the present, of taxonomy and systematics, of the idea of evolution, and of the various theories of inheritance up to the modern discovery of the diversity of DNA. The prose is usually excellent, though in a few respects American misusages have been appropriated by Mayr rather too thoroughly. Not surprisingly, American has some of the characteristics of a pidgin English, including the inability to distinguish between words of similar sound, however important and useful their distinctions of meaning; and if the proofs I have worked from are any guide, there is a near-total confusion between alternate and alternative, masterful and masterly, etc. Prepositions also suffer somewhat ("grist on the mill"). Equally American is his sur-

The Growth of Biological Thought: Diversity, Evolution, and Inheritance. By Ernst Mayr. Pp. 896. ISBN 0-674-36445-7. (Harvard University Press: 1982.) \$30, £21.

prise over Darwin's procrastination — "one would think that he would rush this, the most important theory in biology, to the printer as quickly as possible", (p.420). A peculiarly Mayrian trick is constantly to put words in inverted commas (sometimes totally unnecessarily) to warn the reader that he does not mean quite that. But what



exactly then does he mean? There is no place for inverted commas in scientific prose except to mark quotations.

Nevertheless, this is criticism at a high level. There is no dullness in the book and very little obscurity, but for whom was it written? Mayr indicates (p.19) that

it is not technical to the extent that a layperson would have difficulty with the exposition. It is a major advantage of the history of ideas in biology that one can study it without a background knowledge of the name of a single species of animal or plant or of the major taxonomic groups and their classification. However, a student of the history of ideas must acquire some knowledge of the dominant concepts in biology . . .

One can also read *Hamlet* omitting the

Prince (one of Darwin's daughters wanted to omit Falstaff from *Henry IV*). The history of biology without a knowledge of the material generating and checking the concepts is precisely that useless and emasculated history of to-ing and fro-ing of concepts, so tiresome and uninformative, which is produced when historians and philosophers write histories of biology. At that rate science becomes as bad as philosophy. In science one must not only generate new concepts; they must be shown to be applicable to the real world.

Fortunately Mayr does not act on this ruinous precept. One of the virtues of the work is that it does cope, though not always sufficiently, with what controls that part of biology (or any other science) which really is science, namely the actual materials and phenomena investigated. A glossary of four whole pages is inserted to help the "layperson", but when one finds that mastodon is in it, but not stochastic, *Fragestellung*, deme, ichthyosaur, phenetic, euphorbs, chorda, *Bauplan* or *Urpflanze*, and that quinarian appears on p.102 but is not explained until p.202, Down's syndrome on p.581 with explanation only on p.759, neither being in the glossary, it becomes evident that Mayr is really writing for Simpson, Dobzhansky, Huxley and other professional students. This is not surprising. It is extremely difficult to abandon one's habitual universe of discourse.

Moreover, it is a very good thing that Mayr has not done so.

He remarks himself that historians are not always aware of how complex biological concepts are. It is one of the great strengths of his book that he frequently gives tabulated or listed components of particular theories or concepts, in order to disentangle the numerous themes involved in them. I for one would be delighted if this book serves to put off a whole lot of intellectuals intending to burst into print on evolution and related topics.

In his exposition of his approach, Mayr includes an amusing personal apologia (pp.9-10):

Whenever possible, I have attempted a synthesis of opposing viewpoints (unless one of them is clearly in error). Where the situation is quite unresolved, I have described the opposing viewpoints in categorical, sometimes almost one-

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sided, terms in order to provoke a rejoinder, if such is justified. Because I hate beating about the bush, I have sometimes been called dogmatic . . . [but] This has never been my attitude, and, indeed, I pride myself on having changed my mind on frequent occasions.

When a man gets round to saying such things of himself, his colleagues have usually been saying them for the last 30 years, as I can testify personally; I can also testify personally to Mayr's changes of mind (and his candour in notifying them in print). Those Americans who in spite of their rugged frontiersman images have not had the guts to stand up to him have only themselves to blame. Nevertheless, even Mayr admits that this strategy is debatable. It all depends on whether it generates more heat than light, and a good deal of avoidable heat is going to be produced by critics of the book. Still, it must be admitted that a clear formulation of wrong ideas can be a real help in the advancement of science, as with Lyell (p.495). As Francis Bacon said, we can learn from error, not from confusion. Wynne Edwards has done similar service in his discussion of group selection, as has Popper in the methodology of science. (Mayr does not notice their subsequent retractions.)

Mayr is fully aware — how could he not be? — of his own magistral position in the modern synthesis, and some references to himself, e.g. p.449, are not quite as modest as are his remarks about his own limitations (e.g. pp.7, 400). The limitations a man is aware of, he can do something about; it is those he is unaware of that are serious. Three seem to me particularly notable. One is his lack of experience in handling plants. The book is a zoologist's book, even where plants are mentioned. Some of his harsher remarks about de Vries could not have been written by a botanist, a horticulturist or even a gardener. The varieties one actually finds in plants are either fluctuations or modifications, until a good deal of work has been done on them. "So-called 'difficult' genera like *Rubus* or *Crataegus*" (p.263), are difficult, and the difficulty cannot be surmounted by setting them aside. Similarly Mayr fails to appreciate the strength of botanists' objections to Weismann (which affected some zoologists as well), and his references to polyploidy and allo-tetraploidy in plants (pp.361, 645) are as perfunctory as in his contributions to *The Evolutionary Synthesis* (Harvard University Press, 1980). To call polyploidy a special case in speciation is to provoke botanists in quite the wrong way. They might well ask whether birds and insects are not special since they manifest so little of it. Equally, single-gene bases for sexual isolation have been known for a long time in plants; that only a small part of the genotype is concerned is not such a discovery as Mayr seems to think (p.605).

Secondly, he is not particularly at home among the invertebrates. This matters much less than his neglect of the botanical

side in this book, but occasionally shows, as when he speaks of Lamarck (pp.346, 367) as establishing on his fossil shells "virtually unbroken phyletic series". To anyone who has worked through Lamarck's species in the *Histoire des Animaux sans Vertèbres* this is a misleading exaggeration.

Thirdly, and perhaps most important, he has never worked on natural selection, only on the consequences of it. He misses throughout the book one of the most extraordinary facts about work on evolution, namely that hardly anyone has done anything on natural selection in the wild. Almost every worker has *talked* about it, often at a great length and with subtle mathematics, but very few have had any experience of it actually in action and much of the recent talking has been little better than politico-religious propaganda. If indeed one wishes to confine oneself to concepts, one can go on treating the questions raised virtually as ideological, which also can generate heat, but hardly light. The treatment of the actual role of natural selection, as far as we can understand it up to the present, is one of the weaker features of the book.

But having said this, again at a high level, one must also say that there is no other book like it for range, depth and insight into the nature and development of these three major biological themes, and the methodology and position of biology among the sciences. Particularly good are Mayr's analyses of essentialism and typological thinking as against population thinking, of the differences of interest and approach between those who are fascinated by diversity and by adaptation, of the necessity for both historical types of explanation and immediate causal ones in biology, and of the incompatibility of the approach of experimentalists and naturalists. (One should now add a third category, the computer-user, with knowledge of organisms neither in the wild nor in the laboratory.) By the side of this book, Singer is trivial, Radl a hopeless *a priorist*, Nordenskiöld a religious propagandist.

One could of course list a considerable number of minor, or not-so-minor errors; some are almost inevitable in a book of this scope, but I will confine myself to four, answering Mayr in his own style. (1) p.25: "The Greeks always looked for rational explanations in the world of phenomena". This is pure typological thinking; most did not, and of the few that did, some had a pretty bad time because of it. Read E.R. Dodds's superb *The Greeks and the Irrational* (University of California Press, 1951). (2) pp.361–362: "The Enlightenment ended, so to speak, with the French Revolution . . .". It did not; look at the followers of Paine and Bentham, and Robert Chambers. (And what does "so to speak" mean?) (3) p.510: Huxley has a perfectly explicit reference to natural selection and several indirect ones in his

essay on the reception of the *Origin of Species*; to say that he has none is wrong, and to label him as an essentialist is again typological thinking. And lastly, a more general point (4) pp.375, 504 etc.: A better appreciation of natural theologians and scientists in England is achieved if one considers the reliability of *testimony*; in science, there is one's own experience, the personal witness of acquaintances, and the testimony of others in papers and books. One is unique in this respect in being the testimony of the Author of Creation, and

therefore particularly valuable.

Certain classes of mind, Marxist in particular, will detest this book; most others will find it exceedingly stimulating, not to say infuriating. It has as many points as a porcupine, for intellectual aggression, not defence. There will be a lot of love-hate relationships engendered. I love it, spikes and all. □

A.J. Cain is Derby Professor of Zoology at the University of Liverpool.

High time for unification in catalysis

R.O.C. Norman

Metal-catalyzed Oxidations of Organic Compounds. By Roger A. Sheldon and Jay K. Kochi. Pp.424. ISBN 0-12-639380-X. (Academic: 1981.) \$56, £37.

METAL-catalysed oxidations of organic compounds have been attracting increasing attention over the past decade or so in three conventionally distinct fields of chemistry: liquid-phase oxidations, traditionally an area of study by the organic chemist; heterogeneous gas-phase reactions, the province of the physical chemist; and enzymatic reactions, that of the biochemist. Many of us who are involved in one of these branches of the subject have sometimes been slow to appreciate the value to our own field of an advance in either of the other two, and it was high time someone attempted to unify the subject. Moreover, for obvious reasons, metal-catalysed oxidations have strong attractions to industrial chemists, who can almost certainly be helped in their task by an understanding of how nature works; for example, it is probably not an exaggeration to suggest that a full understanding of how cytochrome P450 acts in the living cell could help an industrial project aimed at catalysing the aerial oxidation of benzene to phenol.

In this book the task of unification has been undertaken by two men who are well suited to it by virtue of both their complementary fields of experience — industry and academia — and their distinguished activities as researchers in the field. They have succeeded admirably.

The structure they have adopted is logical: to consider first the mechanistic principles of the oxidations, grouped by reaction type — oxidations by molecular oxygen, by peroxides, by oxometal reagents and so on; and then to describe synthetic methodology, grouped by compound type — olefins, aromatic hydrocarbons, alkanes, oxygen-containing compounds, and nitrogen, sulphur and phosphorus compounds. It is true that this results in a certain amount of repetition; for example, the mechanism of oxidation

of arenes by cobalt(III) on p.122 is repeated on p.319. This may irritate some readers but, by and large, it serves to reinforce one's knowledge and understanding in proceeding through a complex subject.

The coverage is enormously comprehensive, including details of reactions catalysed by (salts of) 33 metals, with well over a thousand references, including some from 1981. To some extent, then, the account reads more like a review than a textbook. The disadvantage of this is that little, almost trivial, points are occasionally included for completeness and slightly upset the flow and development of the major themes; but this is certainly outweighed by the advantage of having such a valuable collection of well-referenced information so readily to hand.

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Moving pictures

P.W. Hawkes

Image Sequence Analysis. Edited by T.S. Huang. Pp.437. ISBN 3-540-10919-6; 0-387-10919-6. (Springer-Verlag: 1981.) DM85, \$39.60.

PROCESSING a single black-and-white image in a computer requires a substantial amount of memory and, all too often, of time. If we add colour, the difficulties become appreciably but not insuperably worse. As soon as we are driven to try and extract information from large numbers of images, though, the complexity and immensity of the problem become such that all but the very best-equipped must surely blench. Reading between the lines of this interesting and detailed discussion of the problems of extracting information about moving objects from image

sequences, and related topics, I cannot help feeling that Professor Huang and his colleagues must occasionally have quailed; nonetheless the overall impression is one of optimism.

The book is divided into three parts. The first consists of an introduction, largely devoted to the estimation of motion parameters, and a review by H.H. Nagel of the various applications (with an extensive bibliography and an author index). The second, on image sequence coding, enhancement and segmentation, contains four chapters: "Image Sequence Coding" by E. Dubois *et al.*; "Image Sequence Enhancement" (the editor and Y.P. Hsu); "Image Region Extraction of Moving Objects" (B.M. Radig); and "Analysing Dynamic Scenes Containing Multiple Moving Objects" (J.K. Aggarwal and W.N. Martin). The final section, on medical applications, consists of a single chapter on processing medical image sequences by W. Spiesberger and M. Tasto.

The possible applications of this work are, in many cases, of considerable industrial importance, with the result that although much abstract and abstruse material is presented, we are repeatedly brought back down to earth by the specific illustrations. In the introduction, Huang and Tsai offer the following by no means exhaustive list of potential applications: road traffic monitoring; cloud tracking; microcinematography and X-ray sequences of moving parts of the body, the heart in particular; bandwidth compression of picture-phone and TV conferencing signals; robot vision and dynamic monitoring in industry; and, inevitably, target tracking for military purposes. Readers from other fields will have no difficulty in extending this list: *in situ* studies in electron microscopy, the behaviour of nerves and muscles — there can be few fields where studies of rates and types of change could not benefit from some automation.

Most of the book is concerned with image coding and with the study of rates of change of position, ranging from the position of cars or missiles to the position of a contrast medium in a diseased and a normal kidney. It is written in such a way that the newcomer familiar with "static" image processing can understand in detail the methods used to analyse the "dynamic" situation and, at the same time, the absolute beginner — industrialist, tycoon, general, doctor — can comprehend at least the kinds of problems for which the methods have been devised and judge whether they will be of any help to him. In short, this is a meaty, fairly readable and eminently useful addition to the image processing literature. □

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Mineralogy for everyman?

B.E. Leake

The Encyclopedia of Mineralogy. Encyclopedia of Earth Sciences, Vol. IVB. Edited by Keith Frye. Pp.794. ISBN 0-87933-184-4. (Hutchinson Ross: 1982.) \$95, £62.80.

WHAT does the non-mineralogist want to know about mineralogy? This encyclopaedia sets out to provide the answers but of course those answers are determined by what the editor conceives the reader will want to know, and that is difficult to predict.

According to the preface, the book is mainly intended for the non-mineralogist and the advanced professional is expected to "have little use for our volume". The scope is therefore wide, and the references to mineralogical journals and museums are extensive so that inquirers can follow topics to a greater depth than a general survey can provide. Just over 100 writers — most of them in the United States — contribute 140 articles, ranging alphabetically from abrasive materials to zeolites. These are followed by a mineral glossary containing about 3,000 entries of mineral groups, species and varieties, including mineraloids, in which brief descriptions of the chemical formula, physical properties, occurrence and use of each mineral are terminated by references to relevant literature.

In any encyclopaedia the key element is the index. However well organized, erudite or comprehensive the coverage, the information extractable from such a book is controlled by the quality of the index. For mineral names the index is good, but it is inadequate for many other topics on which would-be users could well consult the book. The mineralogical composition of common building bricks or porcelains? Not even the word ceramic appears in the index. Which crystals are used in X-ray fluorescence analysis? What is diffraction? None of these questions can be answered by consulting the index; some of them no doubt lie in other volumes of this mammoth series of earth science encyclopaedias, of which this is only Vol. IVB. Moreover any reference book must have omissions vulnerable to criticism. The mineralogies of cements and glasses are described, however, so the coverage is not restricted to natural occurrences.

In my view the book is too technical for the non-mineralogist. It is three-quarters designed for the professional mineralogist — who will normally prefer more extensive texts or *Mineralogical Abstracts* — although the mineral glossary, which

occupies a quarter of the volume, will be useful as a quick guide into the literature. The information provided about most minerals is that which a *mineralogist* would normally be concerned with, such as optical and crystallographic properties. Items such as chemical properties (as distinct from chemical composition) are rarely considered so that the solubility of minerals in water, the effects of heating minerals together etc. are scarcely dealt with, although eight pages are accorded to the blowpipe analysis of minerals and five to staining techniques, both of which are clearly described. No doubt a major problem for the editor was the lack of available information of an unconventional type, since most data on minerals are provided by mineralogists and the articles were thus largely written by them.

Sex, parthenogenesis and the tangled bank

Mark Ridley

The Masterpiece of Nature. By Graham Bell. Pp.635. UK ISBN 0-85664-753-5; US ISBN 0-520-04583-1. (Croom-Helm/University of California Press: 1982.) £25, \$45.

"SEXUAL generation", wrote Erasmus Darwin, "seems the chef d'oeuvre, the master-piece of nature". Sex is also one of biology's master-problems, and has been honoured by three of the master-works on evolution which have appeared in the last decade: M.T. Ghiselin's *The Economy of Nature and the Evolution of Sex* (California University Press, 1974), G.C. Williams's *Sex and Evolution* (Princeton University Press, 1975) and J. Maynard Smith's *The Evolution of Sex* (Cambridge University Press, 1978). Graham Bell has combined the theorizing and algebra of a Williams and a Maynard Smith, with the comparative biology of a Ghiselin, to produce a book which is, indeed, more than the length of the other three put together. *The Masterpiece of Nature* is a pleasantly written and important work; it can stand with the other three on the shelf, even if it does not edge them over into the wastebasket as its conclusions sometimes imply.

First, some praise for the 170-page review of sexuality which comprises Chapter 3. It is an immense achievement, which systematically reviews the literature on sex and parthenogenesis for all the taxa of multicellular animals. Despite a few minor omissions, it will be of great value as the only available zoological encyclopaedia of sex.

Sex is a puzzle because it has an apparent two-fold disadvantage compared to

Nonetheless if you want to know about piezoelectricity, phantom crystals, geological barometry and thermometry, pleochroic haloes and thermoluminescence, it's there in a potted form. Also Epsom salt and human and vertebrate minerals, but unless you are a crystallographer the article on point groups and the enumeration of the twin laws of the feldspars will surely leave you baffled — the list of the Council members of the International Mineralogical Association from 1958 to 1978 is more readily understood.

The book, then, will be a useful addition to general libraries. But it is too complex for the non-mineralogist and largely repeats information already available in numerous, recently published mineralogical textbooks. □

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asexual reproduction. A mutation which caused a female bearer to reproduce asexually would go into all its offspring rather than just half of them. And the offspring would all be females, which lay eggs, whereas half of the sexual female's offspring are males whose energetic contribution to the next generation is usually nothing. Recent work has mainly attempted to discover some unnoticed advantage to sex sufficiently great to outweigh its huge, two-fold cost.

Bell takes a different line. He is less concerned with thinking up some new theoretical advantage to sex than with finding out which of the existing theories best explains the incidence of sex and parthenogenesis in nature. The facts meet the theories in Chapter 4, and Bell's verdict is that "the problem originally set has been solved" (pp.391–392) in favour of a theory which he calls "the tangled bank". The tangled bank is, in its emphasis, a new theory. It proposes that there is spatial variation of the environment; each species exploits several niches in space. An asexual clone could take over one niche, but then sex may become advantageous. The asexual progeny must compete among themselves within their one, already saturated, niche. The sexual progeny are more diverse, and so are adapted to a greater diversity of niches. Parthenogenesis is, in fact, most common in empty patches and other circumstances of reduced competition.

The tangled bank is closely related to models of what Maynard Smith has called "sib competition". So I was surprised when, in his chapter on the theories, Bell lumps the previous sib competition models

The second volume of the multi-volume treatise *Social Insects*, edited by Henry R. Hermann, has recently been published by Academic Press. Price is \$55, £36.40. (For a review of Vol.I see *Nature* 282, 884; 1979.)

along with some others as "best man" theories, and discusses them separately from the tangled bank. The distortion at this stage is clear, but it does not become damaging until the two resurface as competitors in Chapter 4. To make the sib competition (best man) model into an opponent, he strips it of spatial variation, of temporal variation in all but physical factors, and demands that if sex is an adaptation to changing environments then the organism ought to reproduce sexually in the changed environment. The sickly resultant theory he then bullies with "as rigorous a comparative scrutiny as we can devise" (p.359). Its tongue thus cut out he then scolds it for silence: he "receives no explanation" for one fact after another, and the rhetoric of refutation resounds through the chapter. At the extreme he finds "incompatible with the lottery version of the best man" (p.368) a study which seems to me to fit it rather well: "Hebert has concluded . . . that most populations are founded by only one or a very few ehippia [of *Daphnia*]", which looks very like the sib competition model with a single survivor in each patch. Yet Bell concludes that "this offers little scope for the lottery principle".

Bell has deceived himself by his dichotomy between tangled bank and sib competition (best man) theories. But let him not deceive us as well. We can sap him by colouring back in the caricatured "best man". Then we won't be fooled by all the remorseless "refutation" and "rejection". We can watch Bell's fascinating advocacy of his tangled bank, all the time seeing him make the strongest case ever that some kind of sib competition is the best explanation of the patterns of sex in nature. □

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By any other name

Keith Bell

The Cambridge Encyclopedia of Earth Sciences. Edited by David G. Smith. Pp.496. US ISBN 0-517-54370-2; UK ISBN 0-521-23900-1. (Crown/Cambridge University Press: 1982.) \$34.95, £19.95.

THE word "encyclopaedia" is one of the most awesome in our language, conjuring up visions of dull, pedantic prose, intimidating collections of facts and a morass of unconnected topics. To me, such volumes were always best left alone. Therefore, it was with some trepidation that I started thumbing through the contents of *The Cambridge Encyclopedia of Earth Sciences* expecting the worse. This was not to be.

Between the covers rests an extensive collection of topics, clearly written, beautifully illustrated and surprisingly up to date. Do not be fooled by the title — the only encyclopaedic feature of this book is its breadth and scope. An encyclopaedia it is not. The book is sub-divided into six fairly broad, distinct parts covering 27 topics that start with the history of the earth sciences and end with the geology of the Solar System. Included are accounts of such diverse fields as remote sensing, the development of life on land and seismic reflection profiling. There are no snappy definitions or thumb-nail sketches of your favourite topic here — this volume is definitely not of the "let's see what it says about such and such a subject" variety. Presented instead is a carefully planned, well-thought-out, overview of the earth sciences. Written primarily by members of the Open University, the book seems to be a much modified and markedly improved version of the popular Open University textbook *Understanding the Earth*, first published in 1971.

Its unfortunate billing as an encyclopaedia implies that information can be readily extracted from the book, but neither its format nor content allow this; it may take some time and a little effort to pull together information relevant to one particular topic. This is especially true of mineral deposits; information on the subject is scattered throughout many sections of the book.

As an introduction to the earth sciences this volume is one of the most exciting publications to emerge in the past few years, one worth having for the diagrams alone — many in colour. It is a text that will probably include among its audience scientists outside the field, interested laymen and introductory geology students. Although not aimed at the professional geologist, so much useful information abounds in this one volume that even those actively working in the earth sciences will find something of interest. I would relish recommending this book as a first-year textbook in the earth sciences, but I can already hear the groans as the title is written up on the blackboard.

Nevertheless, someone has had the verve and imagination to pull together a great deal of information about an area of science that is changing at a dazzling speed. Such a massive undertaking must have appeared formidable to any editor, but to plan and create a volume like this in the short span of three years is really quite remarkable. Its welcoming clarity and freshness should do much to intrigue a host of readers about the earth sciences, in addition to providing a quick refresher course to those geologists who, for one reason or another, have fallen behind. But, remember, it is a book that flies under a false flag. □

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Ideas of desert life

J.L. Cloudsley-Thompson

Biology of Desert Invertebrates. By Clifford S. Crawford. Pp.314. ISBN 0-387-10807-6. (Springer-Verlag: 1982.) DM89, \$41.50.

DURING the present century, the study of desert biology has passed through at least three phases. An initial expansion of interest led to an enhanced understanding of the desert biome (exemplified by the late Professor P.A. Buxton's pioneering work, *Animal Life in Deserts*, first published as early as 1923). This was followed in the years after the Second World War by a considerable amount of research on the adaptations, physiology and phenology of desert animals. The third phase began with an awakening of interest in ecosystems, and the establishment of the International Biological Programme in 1964. Less than ten years later, interest in the environment had grown to a point at which another generation of biologists was viewing desert biology from the perspective of the community and the ecosystem. The impetus for this approach came partly from the intellectual and financial backing of the IBP, and partly from major advances taking place in ecological theory and physiological methodology.

Cliff Crawford's valuable statement not only brings together much of the work of past and present investigators, but reflects his own conceptual bias — probing into what desert invertebrates do and how they do it — in dealing with a topic not easily manipulated. His book is organized in five parts: Deserts and Desert Invertebrates; Adaptations to Xeric Environments; Life-History Patterns; Invertebrate Communities; Invertebrates in Desert Ecosystems; Summary Remarks. An idea of the detail included in this synthetic treatment is indicated by the fact that there are no less than 33 pages of references. In the last analysis, despite the recent surge of facts and interpretations, so evident in the book under review, we are plainly still far from a comprehensive understanding of the roles of desert invertebrates. Are not some of their patterns of adaptation, life history and community interactions more apparent than real? Continued study will doubtless reveal the truth in such matters.

Only to the uninitiated do arid regions appear dull and lifeless. Their inhabitants may, indeed, be secretive and few in number, but the biological problems posed by their very existence in such harsh and inhospitable surroundings makes the desert an exciting environment for research. Crawford's vivid text and striking photographs will undoubtedly stimulate the interest of the initiated, and serve as a reference and source of new ideas for research workers. □

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Cancer as deviant differentiation

D.G. Harnden

Cancer Biology. By Raymond W. Ruddon. Pp.344. Hbk ISBN 0-19-502942-9; pbk ISBN 0-19-502943-7. (Oxford University Press: 1982.) Hbk \$21.95; pbk \$14.95, £10.

IT is relatively easy to describe the characteristics of cancer cells but rather more difficult to say which ones are critical for conferring malignancy. Similarly, it is easy to conclude that there must be an alteration in the genome; but whether the change is structural, regulatory or a combination of the two has always seemed rather harder to define. It comes as something of a culture shock, therefore, to find a book which not only assumes that it is the regulatory changes that are fundamental but makes this thesis sound uncomfortably convincing for an advocate of somatic mutation. To quote Dr Ruddon (p.55):

What we need to know to understand carcinogenesis and to develop ways of preventing or curing cancer, then, is contained in the mechanisms of normal cellular differentiation.

It has been clear for some time that an understanding of differentiation could prove to be very important in some cancers, but we now have to reckon with the

possibility that, for the majority of cancers, the principal irregularity is a deviation from, or an arrest at some point in, the pathway of development of the stem cell into a mature differentiated cell. The association between mutagenesis and carcinogenesis, however, is too strong to be unimportant; but it may be that the link lies in the structure and function of regulatory sequences, whose potential for controlling the balance between preparation for cellular proliferation and the production of specific products of the differentiated cell we are only just beginning to understand.

Dr Ruddon does not go out of his way to advocate the importance of regulatory

phenomena. Indeed in later chapters he deals quite fully and fairly with the role of mutational events. Some might call the overall presentation unbalanced, but I found it an interesting and stimulating approach in which the author's personal views and areas of interest feature quite prominently. This makes it much more compelling to read than a multi-author book and makes the breadth of coverage quite remarkable. Little prior knowledge of cancer nor indeed of complex biological techniques is assumed, and this makes it a very good book for senior students and those entering research projects in this field — as well as refreshing for those more fixed in their ways. □

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Ecological theory meets mycological data

C.T. Ingold

The Fungal Community: Its Organization and Role in the Ecosystem. Edited by Donald T. Wicklow and George C. Carroll. Pp.855. ISBN 0-8247-6956-2. (Dekker: 1981.) SwFr. 280, \$98.50.

IN RECENT years mycologists have become increasingly interested in the life processes of fungi in their natural environments, and, at the same time, there has been a growing awareness amongst ecologists of the importance of fungi in the major ecosystems. The mycologist has tended to focus attention on specific niches in which fungi are the dominant organisms; in particular much attention has been given to fungi in the soil. However, the outstanding need is for valid quantitative estimates of the contributions of fungi to the dynamic equilibrium of such major plant communities as forest, prairie or heath. The ecologist is concerned with all of the components of these communities and how they interact. By their very nature, the fungi are perhaps the most difficult to handle.

The Fungal Community, as Swift says in his foreword, is an evangelistic work. It has the worthy aim of stimulating mycologists and ecologists to concerted and integrated endeavour. No less than 52 authors, including some general ecologists, have contributed 41 chapters which are divided into eight parts: fungal ecology and ecological theory; fungal populations; organization of fungal communities; interactions among fungal populations; fungal community development; fungal productivity in ecosystems; fungi in nutrient re-cycling; and finally the aquatic hyphomycete community as an example of how mycologists have approached a specific ecological problem.

The individual chapters are of diverse nature and quality. Some are excellent such as Wicklow on the coprophilous community; Emerson and Natvig on adaptation of fungi to stagnant waters; Frankland on fungal succession; Starmer on yeasts associated with cacti and spread by species of *Drosophila*; Lindeberg on nitrogen cycling in coniferous forests; and Suberkropp and Klug on degeneration of leaves by aquatic hyphomycetes. However, a number of chapters are mediocre. In all, one is left with the feeling that if, instead of 52 authors, a quarter of that number under closer editorial control had contributed, a more valuable and influential work might have resulted.

Three chapters deal with lichens, but the value of considering them in this book is doubtful. As Pike, in his chapter on lichen biomass, remarks "the thallus of lichens bears more similarity to the body of a green plant than it does to the mycelium of most fungi".

Nonetheless, *The Fungal Community* will be read with interest not only by mainstream ecologists concerned with the whole ecosystem, but also by the growing number of mycologists who are taking an ecological approach that is at the same time both experimental and quantitative. (Incidentally it is to be hoped that, in adopting and adapting ecological methods, these mycologists will be at pains to avoid indulging in too much ecological jargon.) The path ahead in the study of the ecology of fungi will undoubtedly be beset with difficulty, but the production of this book is one step forward.

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